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(54) Title: ASSAYS, METHODS AND MEANS

(57) Abstract: The present invention relates to proteins of the conserved Rhomboid family, which are involved in various signalling pathways within cells. Rhomboid proteins are found to possess a novel serine protease activity which cleaves within the transmembrane domain of a polypeptide substrate. Methods and uses of this activity are provided.

Assays, Methods and Means

The present invention relates to proteins of the Rhomboid family, which are conserved throughout evolution and which, in Drosophila, are involved in epidermal growth factor receptor signalling. In particular, the present invention relates to the activity and function of the members of this protein family.

Rhomboid-1 is a member of a group of seven related proteins in Drosophila, each with seven TMDs, and is the prototype of a family conserved throughout evolution (Wasserman et al., (2000) Genes Dev. *14*, 1651-1663). Although no activity or function has been previously assigned to any member of this family, Rhomboid-1 appears to be the principal trigger of epidermal growth factor receptor (EGFR) activation in Drosophila.

EGF receptor tyrosine kinases regulate many cellular decisions in animal growth and development. Drosophila has a single EGF receptor, which is equally similar to all four of the mammalian ErbB receptors and probably represents their evolutionary prototype. Like its mammalian counterparts, the Drosophila EGF receptor has multiple functions during development, including control of differentiation, proliferation and cell survival (Schweitzer and Shilo,(1997) Trends in Genetics *13*, 191-196; Domínguez et al.,(1998) Current Biol. *8*, 1039-1048).

The EGF receptor pathway has been well conserved between flies and mammals and components involved in the mechanism and control of mammalian ErbB signalling (Casci and Freeman, (1999) Cancer and Metastasis Rev. *18*, 181-201) may be identified by Drosophila genetics. This is an important goal

as not only do these receptors regulate many cellular functions in mammals, but their hyperactivity is also strongly implicated in human cancer and other diseases (Yarden and Sliwkowski, (2001) Nature Reviews Molecular and Cell Biology *2*, 127-137).

The principal activating ligand of the Drosophila EGF receptor is Spitz, which is similar to mammalian TGFα and is synthesised with a single transmembrane domain (TMD) and one extracellular EGF domain (Rutledge et al.(1992) Genes Dev. *6*, 1503-1517). Although genetic evidence has led to the suggestion that Spitz may be proteolytically cleaved to a soluble extracellular fragment in order to function as a ligand, this has not been shown biochemically (Freeman, 1994 Mech. Dev. *48*, 25-33; Schweitzer et al., (1995) Genes Dev. *9*, 1518-1529.; Golembo et al.(1996) Development *122*, 3363-70).

Although Spitz was initially identified genetically, its molecular mechanism was strongly suggested by its similarity to known mammalian ligands. This is not true for other EGF receptor signalling components which have been discovered by fly genetics. For example, the transmembrane molecules Rhomboid-1 and Star are genetically defined as primary regulators of EGF receptor signalling in Drosophila but no function is suggested by their protein sequences.

Rhomboid-1 and its close homologue, Rhomboid-3, are required for EGF receptor activation; in many contexts they trigger ectopic activation of the pathway; and finally, the expression pattern of the *rhomboid-1* gene prefigures receptor activity (Bier et al., (1990) Genes Dev. *4*, 190-203; Freeman et al., (1992) Development *116*, 335-346; Ruohola-Baker et al., (1993) Cell *73*, 953-965.; Sturtevant et al., (1993) Genes Dev. 7, 961-973; Golembo et al., (1996) Development *122*, 3363-70; zür Lage et al. (1997) Current Biology 7, 166-175; Wasserman and

Freeman, (1998) Cell *95*, 355-364; Guichard et al., (1999) Development *126*, 2663-76; Wasserman et al., (2000) Genes Dev. *14*, 1651-1663).

Similar results obtained with Star, a type 2 transmembrane protein with a single TMD (Kolodkin et al. (1994) Development *120*, 1731-1745.), suggest that it also regulates EGF receptor signalling in most contexts. Genetic analysis indicates that Rhomboid-1 and Star both act in the signal-emitting cell (Heberlein et al., (1993) Devl. Biol. *160*, 51-63; Golembo et al., (1996) supra; Guichard et al., (1999) supra; Pickup and Banerjee, (1999) Dev. Biol. *205*, 254-259; Bang and Kintner, (2000) Genes Dev *14*, 177-86; Wasserman et al., 2000 supra).

Despite being such important regulators of EGF receptor activation, nothing has been reported about the molecular function of Rhomboid-1 and Star. Although a role in the production or presentation of ligands seems likely, other proposals have included roles in adhesion or promoting active signalling complexes in the plasma membrane (reviewed in Wasserman and Freeman, (1997) Trends in Cell Biol. 7, 431-436).

In the light of evidence for Spitz cleavage (Freeman, (1994) Mech. Dev. *48*, 25-33; Schweitzer et al., (1995) Genes Dev. *9*, 1518-1529), one model has been that Rhomboid-1 somehow promotes this proteolysis (Golembo et al., 1996 supra), although the lack of recognisable protease domains (Bier et al., 1990 supra) suggests that Rhomboid-1 may not be the protease itself. Indeed, the mammalian homologue of Spitz, TGFα, is proteolytically cleaved by TACE, an ADAM family metalloprotease, which has fly homologues (as yet genetically uncharacterised) and which is unrelated to Rhomboid-1 (Peschon et al., (1998) Science *282*, 1281-4). Furthermore, recent evidence has shifted the emphasis towards a role for Star and

Rhomboid-1 in ligand presentation at the cell surface (Guichard et al., 1999 supra; Bang and Kintner, 2000 supra; Klämbt, (2000) Curr Biol *10*, R388-91). Most directly, Bang and Kintner (2000) have used a Xenopus explant assay to conclude that Rhomboid-1 and Star are only indirectly involved in the proteolysis of Spitz, and that their direct role is to alter the conformation and/or presentation of Spitz at the plasma membrane.

The present invention is concerned with the determination of the biological activity of proteins of the Rhomboid family through the study of the mechanism by which Rhomboid-1 and Star control EGF signal activation.

The present inventors have discovered that proteins of the Rhomboid family are a new class of intra-membrane serine proteases, which act on a range of physiological substrates, including EGFR ligands, such as Spitz. The specificity of the proteolytic activity provides indication that molecules which inhibit these proteins may produce specific and highly significant pharmacological effects.

In Drosophila, full-length Spitz protein is tightly held in the endoplasmic reticulum (ER) until Star chaperones it to the Golgi apparatus. Contrary to previous assumptions, Rhomboid-1 is shown to be localised in the Golgi apparatus rather than the plasma membrane and directly cleaves Spitz to produce a soluble fragment which binds EGFR.

One aspect of the present invention provides a fragment of a Rhomboid polypeptide wherein the fragment proteolytically cleaves a polypeptide substrate.

A polypeptide substrate may be cleaved within a transmembrane domain.

A fragment of a Rhomboid polypeptide may consist of fewer residues than the full-length Rhomboid polypeptide. For example, a fragment of the Rhomboid-1 polypeptide may consist of less than 355 amino acid residues as described herein.

A suitable substrate may comprise a transmembrane domain which includes a five residue motif which has an equivalent conformation, structure or three dimensional arrangement to that of residues 140-144 of the Drosophila Spitz sequence (IASGA). More preferably, such a substrate may comprise a seven residue motif which has an equivalent conformation, structure or three dimensional arrangement to that of residues 138-144 of the Drosophila Spitz sequence (ASIASGA).

Such a polypeptide substrate may comprise a transmembrane domain (TMD) motif which includes one or more of residues 140-144 (IASGA), more preferably 138-144 of the Drosophila Spitz sequence (ASIASGA). Such a TMD motif may preferably include three or more, four or more, five or more, six or more, or all seven such residues. Preferably the TMD comprises at least the GA motif corresponding to residues 143 and 144 of Spitz. As described above, the substrate is cleaved by the Rhomboid polypeptide within the transmembrane domain.

Other suitable polypeptide substrates may comprise a transmembrane motif which has none of the residues of the Drosophila Spitz ASIASGA motif, but which instead possess a motif having an equivalent structure which is cleaved by Rhomboid polypeptide (e.g. Gurken, Keren).

For example, a suitable polypeptide substrate may include an amino acid sequence consisting of the transmembrane region of Drosophila Spitz polypeptide (residues 139 to 164), Gurken,

Keren, or other EGFR ligand exemplified in Table 2 or a variant, allele, derivative, homologue, or mutant thereof.

A variant, allele, derivative, homologue, or mutant may consist of a sequence having greater than about 50% sequence identity with the transmembrane region of the polypeptide, greater than about 60%, greater than about 70%, greater than about 80%, greater than about 90%, or greater than about 95%. The sequence may share greater than about 70% similarity with the sequence of the transmembrane domain of the polypeptide, greater than about 80% similarity, greater than about 90% similarity or greater than about 95% similarity. Preferably, such a variant, allele, derivative, homologue, or mutant comprises residues 141-144 of the Drosophila Spitz sequence (IASGA) or residues with an equivalent secondary structure or conformation, more preferably residues 138-144 of the Drosophila Spitz sequence (ASIASGA) or residues with an equivalent secondary structure or conformation.

The polypeptide substrate may, for example, be an EGFR ligand, such as Spitz, Gurken, Keren or other EGFR ligand exemplified in Table 2 or a chimeric substrate comprising amino acid residues from two or more EGFR ligands.

Other suitable substrates may be selected from the group consisting of the *S. cerevisiae* polypeptides PET100/YDR079W, OSM1/YJR051W, MGM1/YOR211C, MCR1/YKL150W and CCP1/YKR066C, in particular the group consisting of MGM1/YOR211C and PET100/YDR079W.

A Rhomboid polypeptide fragment consists of fewer amino acid residues than said full-length polypeptide. Such a fragment may consist of at least 255 amino acids, more preferably at least 300 amino acids. Such a fragment may consist of 325

amino acids or less, 300 amino acids or less, or 275 amino acids or less.

Such a fragment preferably comprises residues R152, G215, S217 and H281, more preferably residues W151, R152, N169, G215, S217 and H281, which are important for the catalytic activity of the protein and are highly conserved in the Rhomboid family. A suitable polypeptide fragment may comprise amino acid residues 90 to 328 of the full length Drosophila Rhomboid-1 sequence. For example, a polypeptide fragment may comprise residues 90 to 355 of the Rhomboid-1 protein and lack the N terminal cytoplasmic domain of the full length protein or may comprise residues 1 to 328 and lack the C terminal lumenal domain of the full-length protein.

A conserved motif GXSG (where X may be any amino acid residue) is frequently found around the active site serine residue(S217), and a Rhomboid polypeptide preferably comprises such a motif, although variants at position 4 exist. In particular, the motif GASG may be present.

Amino acid residues of Rhomboid polypeptides are described in the present application with reference to their position in the Rhomboid-1 sequence. It will be appreciated that the equivalent residues in other Rhomboid polypeptides may have a different position and number, because of differences in the amino acid sequence of each polypeptide. These differences may occur, for example, through variations in the length of the N terminal domain. Equivalent residues in Rhomboid polypeptides are easily recognisable by their overall sequence context and by their positions with respect to the Rhomboid TMDs.

A Rhomboid polypeptide may also comprise additional amino acid residues which are heterologous to the Rhomboid sequence. For

example, a fragment as described above may be included as part of a fusion protein, e.g. including a binding portion for a different ligand.

A Rhomboid polypeptide suitable for use in accordance with the present invention may be a member of the Rhomboid family or a mutant, homologue, variant, derivative or allele thereof. Suitable polypeptides may have a sequence of Drosophila Rhomboid 1, 2, 3 or 4, Human RHBDL-1 (Human Rhomboid-1: Pascall and Brown (1998) FEBS Lett. 429, 337-340), Human RHBDL-2 (NM_017821), Human RHBDL-3 (Figure 8), Zebrafish RHBDL2 (Figure 11) E. coli glgG, B. subtilis ypqP, P. stuartii A55862 gene product, P. aeruginosa B83259 gene product, S. cervisiae YGR101w and S. cervisiae YPL246c or other polypeptide as exemplified in Table 1.

Other suitable Rhomboid polypeptides may be found in public domain databases, for example by Blast searching or by an annotation indicating the presence of a rhomboid domain.

A polypeptide which is a member of the Rhomboid family or which is an amino acid sequence variant, allele, derivative or mutant thereof may comprise an amino acid sequence which shares greater than about 18% sequence identity with the sequence of Drosophila Rhomboid-1, greater than 25%, greater than about 35%, greater than about 40%, greater than about 45%, greater than about 55%, greater than about 65%, greater than about 70%, greater than about 80%, greater than about 90% or greater than about 95%. The sequence may share greater than about 30% similarity with Drosophila Rhomboid-1, greater than about 40% similarity, greater than about 50% similarity, greater than about 60% similarity, greater than about 70% similarity, greater than about 80% similarity or greater than about 90% similarity. Preferably, an amino acid sequence variant, allele, derivative or mutant of a polypeptide of the

Rhomboid family retains Rhomboid activity i.e. it proteolytically cleaves a EGFR ligand transmembrane domain substrate.

Sequence similarity and identity is commonly defined with reference to the algorithm GAP (Genetics Computer Group, Madison, WI). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, the default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4.

Use of GAP may be preferred but other algorithms may be used, e.g. BLAST (which uses the method of Altschul *et al.* (1990) *J. Mol. Biol.* **215**: 405-410), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* **85**: 2444-2448), or the Smith-Waterman algorithm (Smith and Waterman (1981) *J. Mol Biol.* ***147***: 195-197), or the TBLASTN program, of Altschul et al. (1990) supra, generally employing default parameters. In particular, the psi-Blast algorithm (Nucl. Acids Res. (1997) 25 3389-3402) may be used.

Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Particular amino acid sequence variants may differ from a known Rhomboid polypeptide sequence as described herein by insertion, addition, substitution or deletion of 1 amino acid, 2, 3, 4, 5-10, 10-20 20-30, 30-50, or more than 50 amino acids.

Sequence comparison may be made over the full-length of the relevant sequence described herein, or may more preferably be over a contiguous sequence of about or greater than about 20, 25, 30, 33, 40, 50, 67, 133, 167, 200, 233, 267, 300, 333, or more amino acids or nucleotide triplets, compared with the relevant amino acid sequence or nucleotide sequence as the case may be.

A polypeptide which is a member of the Rhomboid family preferably comprises catalytic residues R152, G215, S217 and H281, more preferably catalytic residues W151, R152, N169, G215, S217 and H281. The presence of these conserved residues may be used to identify Rhomboid polypeptides.

Preferably, a Rhomboid polypeptide comprises at least 5 TMDs, with residues N169, S217 and H281 each occurring in different TMD at about the same level in the lipid membrane bilayer. Preferably, a Rhomboid polypeptide also comprises a GxSG motif, as described above.

A polypeptide which is a member of the Rhomboid family may also be identified by the presence of a Rhomboid homology domain, as defined by the PFAM protein structure annotation project (Bateman A. et al (2000) The Pfam Protein Families Database Nucl. Acid. Res. 28 263-266). The Pfam rhomboid homology domain is built from a Hidden Markov Model (HMM) using 26 rhomboid sequences as a seed. The Pfam 'rhomboid' domain has the pfam specific accession number PF01694.

Other methods suitable for use in identifying Rhomboid polypeptides are well-known in the art.

Particularly valuable methods include the use of Hidden Markov Models built from groups of previously identified Rhomboid

proteins, including, but not limited to Drosophila Rhomboids 1-4. Such bio-informatics techniques are well known to those skilled in the art (Eddy S. R. Curr. Opin. Struct. Biol. 1996 6(3) 361-365). Examples of the use of bioinformatics techniques to identify bacterial Rhomboid polypeptides which are then validated by biochemical analysis are provided below.

An EGFR ligand which is a substrate for a Rhomboid polypeptide, is a polypeptide ligand which binds to EGFR. Suitable ligands may include Spitz, Gurken, Keren or other EGFR ligands which are exemplified in Table 2 and homologues, variants, mutants, alleles or derivatives thereof. A EGFR as described herein may, for example, be a Drosophila EGFR or a mammalian EGFR.

The present analysis of the mechanism and structure of Rhomboid has led to the discovery of a previously unknown gene (RHBDL3) in the human genome which encodes a Rhomboid polypeptide. This gene occupies 68kb on chromosome 17 between the annotated genes NJMU-R1 and FLJ11040 (contig NT_010799). The protein sequence of RHBDL3 is shown in figure 8 and the encoding nucleic acid sequence in figure 7. The present inventors have also identified and cloned a Zebrafish RHBDL2 gene.

In various aspects, present invention provides an isolated nucleic acid encoding a Rhomboid polypeptide which consists or comprises the amino acid sequence shown in Figure 8 or figure 11.

The coding sequence may be that shown included in Figure 7 or figure 10 it may be a mutant, variant, derivative or allele of the sequence shown. The sequence may differ from that shown by a change which is one or more of addition, insertion,

deletion and substitution of one or more nucleotides of the sequence shown. Changes to a nucleotide sequence may result in an amino acid change at the protein level, or not, as determined by the genetic code.

Thus, nucleic acid according to the present invention may include a sequence different from the sequence shown in Figure 7 or figure 10 yet encode a polypeptide with the same amino acid sequence.

An isolated nucleic acid may share greater than about 55% sequence identity with the nucleic acid sequence of Human RHBDL3 as shown in figure 7 or the Zebrafish RHBDL2 sequence shown in figure 10, greater than 60%, greater than about 70%, greater than about 80%, greater than about 90%, or greater than about 95%. A nucleic acid may share greater than about 65% similarity with Human RHBDL3 or Zebrafish RHBDL2, greater than about 70% similarity, greater than about 80% similarity, greater than about 90% similarity or greater than about 95% similarity.

The present invention also extends to nucleic acid that hybridizes with the sequence shown in figure 7 or figure 10 under stringent conditions. Suitable conditions include, e.g. for detection of sequences that are about 80-90% identical suitable conditions include hybridisation overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1 X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

The present invention also includes fragments of such sequences, for example a fragment of the nucleotide sequence of Figure 7 or figure 10. Suitable fragments may consist of less than 1320 nucleotides, for example from 10, 20, 30, 40 or 50 nucleotides to 1200, 1300, 1305 or 1310 nucleotides. Such a fragment may encode a Rhomboid polypeptide as described herein or may be useful as an oligonucleotide probe or primer. In some embodiments of this aspect of the invention, a fragment of the sequence of Figure 7 or figure 10 does not include the published nucleotide sequence with the accession number BE778475.

Another aspect of the present invention provides an isolated Rhomboid polypeptide encoded by a nucleic acid sequence described above, for example the nucleic acid sequence of Figure 7 or Figure 10. Such a Rhomboid polypeptide may comprise or consist of the RHBDL3 amino acid sequence shown in Figure 8 or the RHBDL2 sequence of Figure 11.

An isolated Rhomboid polypeptide may share greater than about 70% sequence identity with the amino acid sequence of Human RHBDL3 shown in Figure 8 or the Zebrafish RHBDL2 sequence of Figure 11, greater than 80%, greater than about 90%, greater than or greater than about 95%. A Rhomboid polypeptide may share greater than about 70% similarity with Human RHBDL3 or Zebrafish RHBDL2, greater than about 80% similarity, greater than about 90% similarity, or greater than about 95% similarity.

Sequence similarity and identity are discussed elsewhere herein.

The KDEL ER retention signal is not found in natural Rhomboid polypeptides and directs the expressed Rhomboid polypeptide to

be retained the ER (endoplasmic reticulum) rather than the Golgi apparatus. As described below, Rhomboid polypeptides labelled with an ER retention signal such as KDEL are particularly useful in assay methods of the present invention, as proteolyic cleavage by such polypeptides is independent of the trafficking activity of the Star polypeptide. This overcomes potential problems with variations in secretion efficiency.

Another aspect of the present invention thus provides an isolated Rhomboid polypeptide as described above comprising an N terminal ER retention signal sequence. A suitable signal sequence consists of the amino acid sequence KDEL.

Such a Rhomboid polypeptide may comprise an N terminal signal sequence consisting of the amino acid sequence KDEL and a Rhomboid amino acid sequence as described herein, for example a sequence of one of Drosophila Rhomboids 1 to 4, RHBDL-1, RHBDL-2, RHBDL-3, *E. coli* glpG, *Providencia stuartii* A55862, *Pseudomonas aeruginosa* B83259 or other member of the Rhomboid family as exemplified in Table 1.

Another aspect of the present invention provides a nucleic acid encoding a Rhomboid polypeptide as described above. Such a nucleic acid may comprise or consist of a nucleotide sequence described herein.

The skilled person can use the techniques described herein and others well known in the art to produce large amounts of polypeptides and peptides, for instance by expression from encoding nucleic acid.

Peptides can also be generated wholly or partly by chemical synthesis. The compounds of the present invention can be

readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Alanine scans are commonly used to find and refine peptide motifs within polypeptides. This involves the systematic replacement of each residue in turn with the amino acid alanine, followed by an assessment of biological activity. This enable the residues responsible for the activity to be determined.

A "derivative" or "variant" of a polypeptide may include a polypeptide modified by varying the amino acid sequence of the protein, e.g. by manipulation of the nucleic acid encoding the protein or by altering the protein itself. Such derivatives of the natural amino acid sequence may involve one or more of insertion, addition, deletion or substitution of one or more amino acids, which may be without fundamentally altering the qualitative nature of the proteolytic activity of the wild type Rhomboid polypeptide.

Functional mimetics of active fragments of the Rhomboid, Star and EGFR ligand polypeptides provided (including alleles, mutants, derivatives and variants) may also be used in methods of the present invention. The term "functional mimetic" means a substance which may not contain an active portion of the relevant amino acid sequence, and probably is not a peptide at all, but which retains, in qualitative terms, a biological activity of natural Rhomboid, Star or EGFR ligand polypeptide. The design and screening of candidate mimetics is described in detail below.

The isolated and/or purified polypeptide or polypeptide fragment may be used in formulation of a composition, which may include at least one additional component, for example a pharmaceutical composition including a pharmaceutically acceptable excipient, vehicle or carrier.

A composition including a polypeptide or polypeptide fragment according to the invention may be used in prophylactic and/or therapeutic treatment as discussed below.

Various aspects of the present invention relate to screening and assay methods and means, and substances identified thereby, for example, assays for substances which inhibit interaction between a Rhomboid polypeptide of the invention and a polypeptide substrate or between a Star polypeptide and a polypeptide substrate. The polypeptide substrate may be an EGFR ligand.

Further assays are for a compound or substance which interacts with or binds a Rhomboid polypeptide and modulates i.e. increases, stimulates, reduces, inhibits or abolishes, its protease activity.

An assay method for identifying a modulator of Rhomboid polypeptide may include bringing into contact a Rhomboid polypeptide as described herein and a test compound, determining binding of the test compound to the Rhomboid polypeptide and determining the protease activity of the Rhomboid polypeptide in the presence and absence of a test compound which binds the Rhomboid polypeptide. Protease activity may be determined by determining the cleavage of a substrate as described below. The Rhomboid polypeptide may be isolated or comprised in a liposome or cell.

A method of screening for and/or obtaining a substance which modulates activity of a Rhomboid polypeptide may include contacting one or more test substances with the Rhomboid polypeptide in a suitable reaction medium, determining the activity of the treated polypeptide and comparing that activity with the activity of the polypeptide in comparable reaction medium untreated with the test substance or substances. The Rhomboid polypeptide may be in the reaction medium in an isolated form or may be comprised in a liposome or cell.

A difference in activity between the treated and untreated polypeptides is indicative of a modulating effect of the relevant test substance or substances, for example, an inhibiting or enhancing effect.

Activity of a Rhomboid polypeptide may be determined by determining the production of proteolytically cleaved substrate. The Rhomboid polypeptide may, for example, act on a membrane-bound substrate to generate a soluble product which is detected.

According to another aspect of the present invention there is provided an assay method for identifying and/or obtaining a modulator of a Rhomboid polypeptide, which method comprises:

(a) bringing into contact an Rhomboid polypeptide and a test compound in the presence of a polypeptide substrate; and

(b) determining proteolytic cleavage of the polypeptide substrate.

A assay method may be carried out under conditions in which the Rhomboid polypeptide normally catalyses proteolytic cleavage of the polypeptide substrate

Cleavage of the substrate may be determined in the presence and absence of test compound. A difference in cleavage in the presence of the test compound relative to the absence of test compound may be indicative of the test compound being a modulator of Rhomboid protease activity.

The Rhomboid polypeptide may be a member of the Rhomboid like family or a mutant, variant or allele thereof. Suitable polypeptides may have a sequence of one of Drosophila Rhomboid 1, Drosophila Rhomboid 2, Drosophila Rhomboid 3, Drosophila Rhomboid 4, Human RHBDL-1, Human RHBDL-2 and Human RHBDL-3, E. coli glgG, B. subtilis ypqP, P. stuartii A55862 gene product, P. aeruginosa B83259 gene product, S. cervisiae YGR101w and S. cervisiae YPL246c or other member of the Rhomboid family as exemplified in Table 1.

Any polypeptide substrate which is proteolytically cleaved by a Rhomboid polypeptide may be used in an assay method as described herein. Such substrates are readily identified using standard techniques. A suitable polypeptide substrate may comprise a transmembrane domain having a lumenal portion which has the same conformation as Spitz residues 140-144 (IASGA),

more preferably the same conformation as Spitz residues 138-144 (ASIASGA). Such a lumenal portion may comprise or consist of Spitz residues 140-144 (IASGA), more preferably Spitz residues 138-144 (ASIASGA). The substrate may comprise a Spitz transmembrane region or a variant, allele, derivative, homologue, or mutant thereof as described above. The polypeptide substrate may be an EGFR ligand, such as an EGFR ligand shown in Table 2.

A suitable substrate may comprise a detectable label such as green fluorescent protein (GFP), luciferase or alkaline phosphatase. This allows convenient detection of the soluble cleaved product and is particularly useful in automated assays.

In preferred embodiments, a substrate does not require the presence of Star polypeptide in order to be cleaved by Rhomboid.

EGFR ligands suitable for use in the present assays are well characterised in the art and may have a structure comprising one or more Epidermal Growth Factor (EGF) domains and a single trans-membrane domain (Groenen L. et al Growth Factors 1994 11(4) 235-257).

Preferably, suitable EGFR ligands have greater than 50% homology, greater than 60% homology, greater than 70% homology, greater than 80% homology greater than 90% homology or greater than 95% homology to a vertebrate EGFR ligand as shown in Table 2. EGF domains may also be identified using pfam (Pfam Accession Number for 'EGF-like domain': PF00008) as described above.

Suitable ligands include Spitz, Gurken, Vein, Keren and variants, mutants, alleles or derivatives thereof. Other examples are shown in Table 2.

In some preferred embodiments, the Rhomboid polypeptide is an RHBDL-2 polypeptide and the polypeptide substrate is a Spitz polypeptide.

A chimeric ligand may have improved properties in methods described herein, for example it may be cleaved more efficiently by a Rhomboid polypeptide, have improved secretion properties or be more readily detected.

Another aspect of the present invention provides a chimeric EGFR ligand comprising comprising sequence from two or more EGFR ligands, for example a chimeric ligand may comprise the transmembrane domain of a first EGFR ligand and the intracellular and extracellular domains of a second EGFR ligand.

A suitable first polypeptide is Spitz and a suitable second polypeptide is TGFα. A chimeric substrate may further comprise a detectable label, such as luciferase, GFP or alkaline phosphatase.

A nucleic acid encoding a preferred chimeric ligand comprises nucleotides 1-130 of the TGFα UTR and signal/propeptide sequence (A of the ATG of TGFα is at 35), a GFP label (nucleotides 131-886), and then the remaining TGFα sequence with the inclusion of Spitz 15aa and TMD (bases 1045-1159).

Assay methods or other methods for obtaining or identifying modulators of Rhomboid activity according to the present

invention may be *in vivo* cell-based assays, or *in vitro* non-cell-based assays.

Methods may be performed in the presence of 10μM Baltimastat (British Biotech) to inhibit the non-Rhomboid dependent shedding of substrate and thereby decrease background.

In *in vitro* assays, the rhomboid polypeptide may isolated or contained in a liposome. Such assays may be performed in the absence of Star polypeptide. Liposome based assays may be carried out using methods well-known in the art (Brenner C. et al (2000) Meths in Enzymol. 322 243-252, Peters et al (2000) Biotechniques 28 1214-1219, Puglielli, H. and Hirschberg C. (1999) J. Biol. Chem. 274 35596-35600, Ramjeesingh, M. (1999) Meths in Enzymol. 294 227-246).

Suitable cell types for *in vivo* assays include mammalian cells such as CHO, HeLa and COS cells.

It is not necessary to use the entire full length proteins for *in vitro* or *in vivo* assays of the invention. Polypeptide fragments as described herein which retain the activity of the full length protein may be generated and used in any suitable way known to those of skill in the art. Suitable ways of generating fragments include, but are not limited to, recombinant expression of a fragment from encoding DNA. Such fragments may be generated by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Small fragments (e.g. up to about 20 or 30 amino acids) may

also be generated using peptide synthesis methods which are well known in the art.

The precise format of the assay of the invention may be varied by those of skill in the art using routine skill and knowledge. For example, interaction between the polypeptides may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. Suitable detectable labels include ^{35}S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein containing an epitope which can be labelled with an antibody.

Fusion proteins may be generated that incorporate six histidine residues at either the N-terminus or C-terminus of the recombinant protein. Such a histidine tag may be used for purification of the protein by using commercially available columns which contain a metal ion, either nickel or cobalt (Clontech, Palo Alto, CA, USA). These tags also serve for detecting the protein using commercially available monoclonal antibodies directed against the six histidine residues (Clontech, Palo Alto, CA, USA).

Preferably, assays according to the present invention take the form of *in vivo* assays. *In vivo* assays may be performed in a cell line such as a yeast strain, insect or mammalian cell line in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into the cell.

In Drosophila, Star chaperones the EGFR ligand from the Endoplasmic Reticulum to the Golgi, where it is cleaved by the Rhomboid-1. In some embodiments, a Star polypeptide may be

used in an *in vivo* assay to deliver an EGFR ligand to the Rhomboid polypeptide.

In assay and other methods according to such embodiments, Rhomboid polypeptide may be contacted with the test compound in the presence of a Star polypeptide. In such methods, the Rhomboid polypeptide, Star polypeptide and EGFR ligand may be present in a cell. This may be achieved, for example by expressing the polypeptides from one or more expression vectors which have been introduced into the cell by transformation.

An assay method for identifying and/or obtaining a modulator of Rhomboid protease may therefore include:

(a) bringing into contact an Rhomboid polypeptide and a test compound in the presence of a Star polypeptide and a EGFR ligand polypeptide; and

(b) determining cleavage of the EGFR ligand.

An assay method may be performed under conditions in which the Rhomboid polypeptide normally catalyses proteolytic cleavage of the EGFR ligand polypeptide.

Cleavage may be determined in the presence and absence of test compound. A difference in cleavage in the presence, relative to the absence of test compound is indicative of the compound being a modulator i.e. an enhancer or inhibitor of Rhomboid activity.

A suitable Star polypeptide may include the Drosophila Star (Database Acc No;SWP:P42519)or a variant, homologue, mutant, allele or derivative thereof. A variant, allele, derivative, homologue, or mutant of Star may consist of a sequence having greater than about 70% sequence identity with the sequence of

Drosophila Star, greater than about 80%, greater than about 90%, or greater than about 95%. The sequence may share greater than about 70% similarity with the sequence of Drosophila Star, greater than about 80% similarity, greater than about 90% similarity or greater than about 95% similarity.

In other embodiments of the present invention, a Star-independent EGFR ligand may be used in cell-based assays method and/or a Rhomboid polypeptide which is retained in the ER, and the use of Star in such methods is therefore unnecessary.

Nucleic acid encoding Rhomboid polypeptides, polypeptide substrates and/or Star polypeptides as described above may be provided as part of a replicable vector, particularly any expression vector from which the encoded polypeptide can be expressed under appropriate conditions, and a host cell containing any such vector or nucleic acid. An expression vector in this context is a nucleic acid molecule including nucleic acid encoding a polypeptide of interest and appropriate regulatory sequences for expression of the polypeptide, in an *in vitro* expression system, e.g. reticulocyte lysate, or *in vivo*, e.g. in eukaryotic cells such as COS or CHO cells or in prokaryotic cells such as *E. coli*. This is discussed further below.

Combinatorial library technology (Schultz, JS (1996) Biotechnol. Prog. 12:729-743) provides an efficient way of testing a potentially vast number of different substances for ability to modulate activity of a polypeptide. Prior to or as well as being screened for modulation of activity, test substances may be screened for ability to interact with the polypeptide, e.g. in a yeast two-hybrid system (which requires

that both the polypeptide and the test substance can be expressed in yeast from encoding nucleic acid). This may be used as a coarse screen prior to testing a substance for actual ability to modulate activity of the polypeptide.

The amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 to 100 nM concentrations of putative inhibitor compound may be used, for example from 0.1 to 10 nM. When cell-based assays are employed, the test substance or compound is desirably membrane permeable in order to access the Rhomboid polypeptide.

Test compounds may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used. A further class of putative inhibitor compounds can be derived from the Rhomboid polypeptide and/or a ligand which binds such as the Spitz TMD. Membrane permeable peptide fragments of from 5 to 40 amino acids, for example, from 6 to 10 amino acids may be tested for their ability to disrupt such interaction or activity. Especially preferred peptide fragments comprise residues 141 to 144 (ASGA) of the Spitz protein, residues 140-144 (IASGA) or residues 138-144 (ASIAGA), or the equivalent regions of other EGFR ligands.

The inhibitory properties of a peptide fragment as described above may be increased by the addition of one of the following groups to the C terminal: chloromethyl ketone, aldehyde and boronic acid. These groups are transition state analogues for serine, cysteine and threonine proteases. The N terminus of a peptide fragment may be blocked with carbobenzyl to inhibit

aminopeptidases and improve stability (Proteolytic Enzymes 2nd Ed, Edited by R. Beynon and J. Bond Oxford University Press 2001).

The present application describes two compounds, TPCK and 3, 4-DCI, which have been shown to inhibit Rhomboid activity. Although these compounds are broad spectrum serine protease inhibitors, they represent examples of lead compounds for the rational design of specific Rhomboid inhibitors.

Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics.

Another aspect of the present invention provides a modulator, for example an inhibitor of Rhomboid protease activity or composition comprising a said modulator, isolated and/or obtained by a method described herein.

Following identification of a substance which modulates or affects polypeptide activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Another aspect of the present invention provides the use of a Rhomboid polypeptide as described herein in a method for obtaining or identifying a modulator, for example an inhibitor, of Rhomboid serine protease activity. Also provided are methods and uses of a Rhomboid polypeptide in the

proteolytic cleavage of the transmembrane domain of a polypeptide substrate.

Rhomboids are involved in quorum sensing - the intercellular signalling carried out by bacteria. Rhomboid inhibitors may be useful in blocking this activity. In one human pathogen (Providencia stuartii) the AarA gene is required to generate a signal in a quorum sensing event (Rather, P. N. et al (1999). J Bacteriol *181*, 7185-7191). The AarA gene encodes a Rhomboid polypeptide (Gallio, M., and Kylsten, P. (2000). Curr Biol *10*, R693-694).

It is shown herein that AarA has the same enzymological activity as Drosophila Rhomboid, is therefore useful in screened for inhibitors which block quorum sensing. Pathogenic bacteria use quorum sensing to influence when to express their toxic virulence factors (for example and review - Zhu, J. et al (2002). Proc Natl Acad Sci U S A *99*, 3129-3134; Miller, M. B., and Bassler, B. L. (2001). Annu Rev Microbiol *55*, 165-199); preventing this signal using a Rhomboid inhibitor would stop these pathogens from being virulent. As the inhibitor does not kill the cells, the selective pressure for the organism to acquire resistance to it will be reduced.

Methods described herein may further comprise the step of determining the ability of said test compound to inhibit the infectivity or virulence of a microbial pathogen. This may, for example, comprise determining the expression of toxic virulence factors in the presence and absence of test compound.

Modulators, in particular inhibitors of Rhomboid activity may be useful in the treatment of pathogen infection, for example

by yeasts and pathogenic bacteria such as Providencia stuartii, E. coli 0157 and Pseudomonas aeruginosa.

Thus, the present invention extends in various aspects not only to a substance identified as a modulator of Rhomboid activity, in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for treatment (which may include preventative treatment) of a pathogenic infection or a condition associated with aberrant ErbB or EGF receptor activity, such as cancer, coronary atherosclerosis, psoriasis, wound healing, survival of premature infants, peripheral nerve injuries/neuropathies, use of such a substance in manufacture of a composition for administration, e.g. for treatment of a pathogenic infection or a condition associated with aberrant ErbB or EGF receptor activity, such as cancer, coronary atherosclerosis, psoriasis, wound healing, survival of premature infants, peripheral nerve injuries/neuropathies, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

A condition associated with aberrant ErbB or EGF receptor activity as described above may also be associated with aberrant Rhomboid activity.

A substance identified as a modulator of polypeptide or promoter function using an assay described herein may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many in vivo pharmaceutical uses. Accordingly, a mimetic or mimick of the substance

(particularly if a peptide) may be designed for pharmaceutical use.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Whilst TPCK and 3, 4-DCI have been shown to inhibit Rhomboid, these compounds lack specificity and so are liable to produce undesirable side-effects, if used therapeutically. They may however represent "lead" compounds for the development of mimetics with improved specificity.

Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound such as TPCK, 3, 4-DCI, or Spitz transmembrane fragments, which have a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn.

The essential catalytic residues of polypeptides of the Rhomboid family are highly conserved and correspond to residues N169, G215, S217, H281, W151 and R152 of the Drosophila Rhomboid-1 sequence. The essential residues

required for cleavage by Rhomboid are residues A141, S142, G143 and A144 of the Spitz sequence. Other important residues include residues A138 S139 and I140 of the Spitz sequence.

Residues which constitute the active region of a peptide or polypeptide are known as its "pharmacophore".

The information provided herein regarding the pharmacophore of the Rhomboid family and its substrate allow their structures to be modelled according their physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process. The discovery of the close relationship between the Rhomboid polypeptide family and the much studied serine proteases provides considerable information regarding the Rhomboid active site.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade in vivo, while retaining the biological activity of the lead compound. The mimetic or mimetics found

by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

For example, mimetics which model the three dimensional conformation of the Rhomboid recognition domain of Spitz (residues 140-144: IASGA, or more preferably residues 138-144: ASIASGA) may be used to screen for a compound which binds and inhibits a Rhomboid polypeptide. Such mimetics may include peptide chloromethyl ketone analogues of the Rhomboid binding domain of Spitz, for example comprising the IASGA or ASIASGA sequence.

Mimetics of substances identified as having ability to modulate Rhomboid polypeptide activity using a screening method as disclosed herein are included within the scope of the present invention.

A polypeptide, peptide or substance able to modulate activity of a polypeptide according to the present invention may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered,

and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous

solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

A convenient way of producing a polypeptide for use in assays and methods according to the present invention is to express nucleic acid encoding it, by use of the nucleic acid in an expression system. Accordingly, the present invention also encompasses a method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally, nucleic acid according to the invention) and testing for Rhomboid protease activity. This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides may also be expressed in *in vitro* systems, such as reticulocyte lysate.

Another aspect of the present invention therefore provides a method of producing a Rhomboid polypeptide comprising:

(a) causing expression from nucleic acid which encodes a Rhomboid polypeptide in a suitable expression system to produce the polypeptide recombinantly;

(b) testing the recombinantly produced polypeptide for Rhomboid protease activity.

Suitable nucleic acid sequences include a nucleic acid sequence encoding a member of the Rhomboid-like family or a mutant, variant or allele thereof as described herein.

A polypeptide may be isolated and/or purified (e.g. using an antibody) for instance after production by expression from encoding nucleic acid (for which see below). Thus, a polypeptide may be provided free or substantially free from contaminants with which it is naturally associated (if it is a naturally-occurring polypeptide). A polypeptide may be provided free or substantially free of other polypeptides.

The recombinantly produced polypeptide may be isolated and/or tested for Rhomboid protease activity by determination of the cleavage of a EGFR ligand polypeptide upon incubation of the polypeptide with the EGFR ligand or other polypeptide substrate.

An isolated nucleic acid as described herein, for example a nucleic acid encoding a Rhomboid polypeptide, may be comprised in a vector. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and

yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*.

Further aspects of the present invention provide a host cell containing heterologous nucleic acid encoding a Rhomboid polypeptide which has a KDEL tag or which is a fragment of a full length Rhomboid sequence and a host cell containing heterologous nucleic acid encoding a Rhomboid polypeptide and an EGFR ligand polypeptide and, optionally, a Star polypeptide.

The nucleic acid may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell.

The introduction of nucleic acid into a host cell, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, tested for Rhomboid protease activity and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

A Rhomboid polypeptide may be co-expressed in a host cell with a substrate polypeptide and the Rhomboid serine protease activity determined by determining cleavage of the substrate polypeptide. Cleavage may be determined by determining the presence or absence of soluble cleavage products which may be secreted into the culture medium.

The principal determinant of cleavability by Rhomboid-1 is the trans-membrane domain of Spitz, in particular the region between residues 138-144 (ASIASGA). A polypeptide comprising a homologous domain is therefore a candidate for being a substrate for a Rhomboid polypeptide. Such polypeptides may be identified by screening databases using standard procedures.

A further aspect of the present invention provides a method of obtaining a substrate for a Rhomboid polypeptide comprising,

(a) providing a test polypeptide,

(b) bringing into contact an Rhomboid polypeptide and the test polypeptide under conditions in which the Rhomboid polypeptide normally catalyses proteolytic cleavage of a substrate; and

(c) determining cleavage of the test polypeptide.

The cleavage of the test polypeptide is indicative of the polypeptide being a Rhomboid substrate.

A test polypeptide may comprise residues 141 to 144 of the Drosophila Spitz sequence or residues with an equivalent three dimensional conformation, more preferably residues 140 to 144 or 138 to 144, or residues with an equivalent three dimensional conformation.

A suitable test polypeptide may comprise the transmembrane region of Spitz (residues 139-164), Gurken, Keren, or other EGFR ligand exemplified in Table 2 or a variant, allele, derivative, homologue, or mutant of such a region.

A variant, allele, derivative, homologue, or mutant of the Spitz transmembrane region may consist of a sequence having greater than about 50% sequence identity with the sequence of residues 139 to 164 of Drosophila Spitz, greater than about 60%, greater than about 70%, greater than about 80%, greater than about 90%, or greater than about 95%. The sequence may share greater than about 70% similarity with the sequence of residues 139 to 164 of Drosophila Spitz, greater than about 80% similarity, greater than about 90% similarity or greater than about 95% similarity.

Suitable test polypeptides may be identified by screening databases using the bioinformatics techniques discussed above.

Another aspect of the present invention provides a fragment of a Rhomboid polypeptide which has no proteolytic activity and which, when expressed in a cell, reduces or abolishes the proteolytic activity of an active Rhomboid polypeptide expressed in the same cell. For example, a fragment consisting of residues 1 to 149 of the Rhomboid-1 sequence, the N terminal cytoplasmic domain, first transmembrane region and part of the first lumenal loop possesses this dominant negative activity.

A further aspect of the present invention is a nucleic acid encoding such a Rhomboid polypeptide fragment.

A dominant negative polypeptide fragment may be used to 'knock out' the activity of endogenous Rhomboid polypeptide as described herein. A host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place *in vivo* or *ex vivo*), may, for example, be a bacteria, archaea, unicellular eukaryote or may be comprised (e.g. in the soma) within an organism which is a fungi, plant or animal, including vertebrates and invertebrates and in particular a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse, rat or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken.

Genetically modified or transgenic organisms comprising such a cell are also provided as further aspects of the present invention. Such animals may be useful in the study of diseases associated with Rhomboid dysfunction.

Another aspect of the present invention provides the use of a dominant negative Rhomboid polypeptide fragment as described herein in an *in vitro* method of inactivating a Rhomboid polypeptide in a cell comprising expressing said polypeptide in said cell.

Aspects of the present invention will now be illustrated with reference to the accompanying figures described below and experimental exemplification, by way of example and not limitation. Further aspects and embodiments will be apparent to those of ordinary skill in the art. All documents mentioned in this specification are hereby incorporated herein by reference.

Figure 1 shows a series of GFP-tagged derivatives of the Spitz/TGFα chimeras and Spitz deletions expressed in COS cells and localised using immunofluorescence.

Figure 2 shows a model of the mechanism of Star and Rhomboid-1 as described in the present application. Spitz is retained in the ER (a) until Star promotes its relocalisation (b) to the Golgi apparatus. There it encounters Rhomboid-1, which induces its cleavage (c), releasing a soluble lumenal fragment. This is then secreted from the cell (d), so that it can activate the EGF receptor.

Figure 3 shows a summary of interactions between Spitz, Star and Rhomboid-1. The chaperoning function of Star is mediated primarily through the lumenal domains of Star and Spitz,

although the cytoplasmic domains contribute to a lesser extent (indicated by arrow). Spitz is otherwise retained in the ER via its cytoplasmic domain. In the Golgi apparatus, the TMD region of Rhomboid-1 induces the cleavage of Spitz within the Spitz TMD.

Figure 4 shows the N and C series of Rhomboid-1 truncations. The N series were triple HA-tagged at the N-terminus and truncated as marked by the arrowheads above the line; the C series were triple HA-tagged at the C-terminus and truncated as marked by the arrowheads below the line. Precise coordinates are shown in Table 3.

Figure 5 shows an alignment of the three closest human rhomboid homologues with Drosophila rhomboid-1: RHBDL-1 (also known as RHBDL), RHBDL-2 and RHBDL-3. In particular the amino acid sequence of RHBDL-3 is shown.

The boundary between the cDNA sequence and predicted sequence of RHBDL3 is indicated. The conserved serine protease motif (GASGG) surrounding the active serine is shown above the sequences; the other catalytic residues we have identified are indicated by arrowheads. Identical residues are shaded black; conservative changes are shaded grey. This alignment was generated with the GCG program 'pileup'.

Figure 6 shows a schematic of Spitz/TGFα chimeras, indicating which retained the ability to be cleaved by Rhomboid-1. Numbered coordinates within the TMDs represent the TMD residues of Spitz that have been replaced by corresponding TGFα residues. The recognition domain for Rhomboid-1 cleavage maps to the lumenal quarter of the TMD, i.e. Spitz residues 140-145.

Figure 7 shows the nucleic acid coding sequence of RHBDL3. The

sequence is predicted by Genemark (and supported by a partial EST [accession number BE778475] indicated by the horizontal line below the sequence).

Untranslated regions are not shown; the sequence begins with the ATG codon predicted to encode the first methionine. Arrows represent positions of introns. Alternating upper/lower case is used to distinguish adjacent exons. This ORF spans 55152 nucleotides of genomic sequence on human chromosome 17 (from contig accession number NT_010799).

Figure 8 shows the amino acid sequence of RHBDL3.

Figure 9 shows the effect of mutation of residues in the Spitz TMD on cleavage efficiency.

Figure 10 shows the nucleotide sequence of the Zebrafish RHBDL2.

Figure 11 shows the amino acid sequence of the Zebrafish RHBDL2.

Table 1 shows a non-exhaustive list of database sequences of Rhomboid polypeptides.

Table 2 shows a non-exhaustive list of database sequences of EGFR ligands.

Table 3 shows the Rhomboid deletion mutants used in the experiments described below.

EXPERIMENTAL

Materials and Methods

Drosophila stocks

The following fly lines were generated by standard techniques: *UAS-mycSpi*, *UAS-Rho-1HA*, *UAS-Rho-1N*, and *UAS-Rho-1*. Other lines used included *MS1096-Gal4* (Capdevila, J. and Guerrero, I. (1994). EMBO J. *13*, 4459-4468.), *hsp70-rho-1* (Freeman, M. et al (1992).Development *116*, 335-346) and a salivary gland specific *Gal4* line (1824 in the Bloomington Stock Centre). All other stocks are listed in Flybase.

dsRNA Interference

RNAi was performed by a modification of the protocol of Kennerdell, J. R. and Carthew, R. W. (Kennerdell, J. R. and Carthew, R. W. (1998). Cell *95*, 1017-26).

100 µg of RNA corresponding to each gene of interest was synthesized by *in vitro* transcription from 5 µg linearized plasmid templates according to manufacturer's instructions (Promega Ribomax system). The resulting RNA was purified using the RNeasy protocol (Qiagen), denatured by boiling, and annealed in 1mM Tris-HCl pH7.4, 1mM EDTA overnight. The resulting dsRNA was ethanol precipitated and resuspended in injection buffer (0.1X PBS) at a concentration of 1-2mg/ml as estimated by agarose gel electrophoresis. Embryos were collected over a 1 hour interval from 2-7 day old cages of *y w* adult flies, placed onto glass slides, dehydrated for 5 minutes, and microinjected laterally through their chorions under Voltalef's 10S oil. Following a 48 hour incubation in a humidified chamber at room temperature, the lethality and the cuticle phenotype were assessed by standard methods.

Construct

Spitz was tagged with a single myc tag between residues 123 and 124 and cloned into the *pUAST* vector for fly transformation. The EGFP ORF (Clontech) was inserted into a BsiWI site between residues 33 and 34 of Spitz. A BsiWI site was introduced by PCR mutagenesis into the N-termini of TGFα and the chimeras containing the TGFα N-terminal domain, allowing the EGFP ORF to be inserted between residues 32 and 33. The Spitz/TGFα chimeras, SpiΔ53C, Spi-15aa and sSpitz, tagged with EGFP ORF in the same position as Spitz, are described elsewhere (Bang and Kintner, 2000 supra, Schweitzer, R. et al (1995). Genes Dev. *9*, 1518-1529.). Spi:TGFα-C includes residues 1-167 of Spitz and 128-160 of human TGFα; it was also EGFP tagged between residues 33 and 34. Rhomboid-1 was tagged at its N-terminus with a triple HA tag and cloned into the *pUAST* and *pcDNA3.1* vectors. A triple myc tag was inserted between residues 3 and 4 of Star or, in a second construct, between residues 83 and 84.

Rho-1N and Rho-1ΔN include residues 1-89 and 89-355, respectively (the first TMD starts at residue 101).The precise coordinates of the Rhomboid-1 N and C terminal truncation series are shown in Table 3. StarΔ291C, Δ266C and Δ47C were made by inserting stop codons at residues 310, 331 and 551, respectively. Unless otherwise noted, all constructs for tissue culture were inserted into *pcDNA3.1* (Invitrogen) except Spi-15aa and spi:TGFα-TMC which were in *pCS2* (Bang and Kintner, 2000 supra).

Spitz Cleavage in Embryos

Embryos were collected over 24 hours from cages with 500-1500 *w; arm-gal4 UAS-mycSpi* females crossed to 100-300 *w; hs-rho1;*

UAS-S males. The resulting embryos were heat shocked at 37°C for 1.5 hours to induce the expression of *rhomboid-1*, and allowed to recover at 25°C for 0-2 hours. The embryos were then dechorionated and lysed in 1ml ice cold RIPA buffer containing a protease inhibitor cocktail (Roche). Insoluble material was removed by centrifugation, and mycSpitz was immunoprecipitated overnight at 4°C with 20µl anti-Myc antibody (9E10, Santa Cruz) directly coupled to agarose beads. The beads were stringently washed in RIPA buffer, resuspended in 20µl SDS sample buffer, and boiled for 5 minutes. MycSpitz was detected by western blot with 1:1000 rabbit anti-myc (A14, Santa Cruz).

Glycosylation analysis in embryos

Embryos were collected as described above and extracts were treated with a variety of deglycosylating enzymes. Endoglycosidase H_f (Endo-H) removes high-mannose N-linked glycans that are the hallmark of ER-resident proteins; peptide-N-glycosidase *F* (*PNGase F*) removes both high mannose glycans and also complex N-linked glycans, typical of Golgi modification; O-glycosidase removes many O-glycans, which are added only in the Golgi; finally, neuraminidase is an exoglycosidase of broad specificity which can improve the efficiency of O-glycosidase. All enzymes were used on denatured samples according to the manufacturers' instructions.

Cell Culture

COS cells were grown in DMEM medium (supplemented with 10% foetal calf serum), and transfected with FuGENE 6 Transfection Reagent (Roche). Cells were transfected in 35mm culture wells with 25-250ng of each construct and empty plasmid to bring the total DNA to 1µg per well. 24-30 hours post-transfection the

medium was replaced with serum-free medium; this was harvested 24 hours later and cells were lysed in SDS-sample buffer. GFP was detected in conditioned medium and cell lysates by western blot with a rabbit polyclonal antibody. For some experiments the serum-free medium was supplemented with the metalloprotease inhibitor batimastat (British Biotech) or ilomostat (Calbiochem).

Yeast strains

YGR101w and YPL246c were C-terminally GFP tagged in the genome directly by PCR (Wigge, P. A. et al (1998). J Cell Biol *141*, 967-977.) in the diploid strain K842 (Nasmyth et al. (1990) Cell *62*, 631-647.). Live cells were imaged on a Radiance Confocal Microscope (BioRAD).

Immunohistochemistry

Cells seeded and transfected on cover slips were fixed for 20 minutes in 4% paraformaldehyde in PBS, and permeabilised for 10 minutes in 0.1% TritonX-100 in PBS. Cells were blocked overnight with 1% BSA, and subsequently incubated at room temperature with primary and secondary antibody for 1.5h and 1h, respectively. GFP fluorescence was often greatly reduced after fixation and required staining with anti-GFP (1:5000) for visualisation. The following primary antibodies were used: mouse anti-Myc 9E10 (Santa Cruz Biotechnology) at 1:250, rat anti-HA (Roche) at 1:500, rabbit anti-PDI (Calbiochem) at 1:250, rabbit anti-Giantin (Seelig et al., (1994) J. Autoimmun. 7, 67-91) at 1:500, mouse anti-p115 (Transduction Labs; a second mammalian cell Golgi marker) at 1:250. Alexa Fluor 568 (red) and Alexa Fluor 488 (green)-conjugated secondary antibodies from Molecular Probes were used at 1:500. Salivary glands were stained according to Munro and Freeman (2000) using 1:400 mouse anti-Drosophila Golgi (Calbiochem) and 1:400 rabbit anti-HA (Y11, Santa Cruz). All fluorescent

images were collected on an MRC Radiance confocal microscope (Biorad).

Protease Inhibitor Assay

Cells were transfected as standard with 1ng of Rhomboid-1 DNA (in 1µg total DNA) and were then incubated in serum-free medium for 24 hours. The medium was then replaced with 0.5ml serum free medium containing protease inhibitor at the indicated concentration and incubated for 1 hour. After 1 hour, the medium was collected, cleared by centrifugation, dialysed overnight against several changes of water and lyophilised. The resulting pellets were re-suspended in SDS sample buffer, boiled and analysed by Western blot. Transfections with a secreted form of Spitz were used in parallel to control for non-specific toxicity of the protease inhibitors or general inhibition of the secretory pathway.

Assay for inhibitors of human Rhomboid (RHBDL2)

HeLa cells are co-transfected with;

(a) RHBDL2 construct ("HAn RHBDL") comprising the RHBDL2 coding sequence with a triple N-terminal HA tag, inserted into the vector pcDNA 3.1+ (Invitrogen), and;

(b) Substrate construct containing the preferred/optimized substrate ("GFP-TGF-Spi-TGF") inserted into pcDNA 3.1+(Invitrogen).

A control vector containing TGF-alpha with an N-terminal GFP tag (with or without a C-terminal HA tag) may be used as a positive control for protein secretion into the medium and is independently transfected into the HeLa cells.

Transfection of construct (b) into HeLa cells in the absence of construct(a) acts as a control for endogenous cleavage of the substrate. Optionally, a metalloprotease inhibitor such as

batimastat may be used to minimize endogenous substrate cleavage in the HTS assay.

Transfected cells are then incubated with test compounds e.g. in a 96-well microplate format.

Supernatants are then collected from the wells and assayed for the presence of GFP in the medium using conventional techniques.

For example, GFP may be captured with a polyclonal or monoclonal antibody, washed and then the captured GFP detected with a polyclonal or monoclonal antibody conjugated to an enzyme (capture ELISA) or with a fluorescent label.

For ELISA, a suitable polyclonal anti-GFP conjugated to horse-radish peroxidase or to alkaline phosphatase is commercially available. Such a conjugate is preferred since the number of incubations required is reduced. Alternatively a biotinylated anti-GFP antibody in combination with an avidin or strepavdin enzyme conjugate could be used.

For a fluorescence assay, Europium- or Terbium-labelled antibody or streptavidin are suitable (e.g. Delphia or Lance reagents, Perkin Elmer). These are labels with a long fluorescence lifetime and can improve the signal:noise ratio.

A variation of the above is to replace GFP in the GFP-TGF-Spi-TGF construct (or to add to the construct) with an enzyme label at the N-terminus to give a direct assay for the cleaved substrate in the medium. Suitable enzymes include Renilla luciferase (Lui,J., and Escher, A. (1999) Gene 237, 153-159) and secretable alkaline phosphatase sequence (SEAP)(Clontech).

Cloning Bacterial Rhomboids

Bacterial Rhomboid genes were cloned by PCR from genomic DNA and inserted into pcDNA3.1 (Invitrogen). Activities were assayed by the standard COS cell assay described above, although typically 100ng of rhomboid DNA was transfected into a 35mm dish of COS cells.

Yeast Knockouts

Knockouts of Saccharomyces cerevisiae rhomboid genes were done by standard procedures (Rothstein RJ. 1983. Methods Enzymol 101, 202-11). Rescue experiments were performed by cloning wild-type or mutated forms of rhomboids into a 2-micron plasmid, which is maintained as a single copy in yeast cells. The plasmid was transformed into the relevant rhomboid knockout cells and its ability to rescue the specific knockout phenotype was assessed.

Zebrafish Knockouts

Knockout of the zebrafish RHBDL2 was performed by standard procedures (McClintock JM, Kheirbek MA, Prince VE. 2002. Development. 129, 2339-2354.). A morpholino antisense oligonucleotide (TCTTGCTCTTCGGTGTCATTATCGC) complementary to the region of the cDNA surrounding the start of translation was injected into 1-4 cell embryos at 2-4μM. After 24 and 48 hours the phenotype was assessed and compared with wild-type embryos of equivalent stages. In situ hybridisation to RHBDL2 was performed by standard techniques.

Results

Spitz is cleaved by a Rhomboid and Star dependent mechanism

Drosophila embryos were investigated for the cleavage of Spitz in response to Rhomboid and Star. UAS-driven Spitz which was myc-tagged near its N-terminus, was ubiquitously expressed in embryos under the control of armadillo-Gal4, in the presence

or absence of ubiquitous Star and/or heatshock inducible Rhomboid-1; tagged Spitz was then immunoprecipitated from embryo extracts (Note that these embryos contain endogenous Star and Rhomboid as well as the ectopically expressed transgenes).

No cleavage of Spitz was induced by Rhomboid-1 alone. Very low level of cleavage was detected in the presence of Star alone, although a new, more slowly migrating species of Spitz appeared, along with a small amount of low molecular weight product.

In embryos expressing Spitz with Star and inducible Rhomboid-1, a truncated form of Spitz appeared in response to the induction of Rhomboid-1 expression. This is the first direct evidence for the cleavage of Spitz in flies, and it demonstrates that proteolysis occurs in response to Rhomboid expression.

This biochemical assay of Spitz activation correlates well with previous genetic evidence showing that Star and Rhomboid are both required for EGF receptor activation and that they act synergistically.

A time course indicated that cleaved Spitz is unstable, declining substantially by 60 minutes after heatshock. Endocytosis participates in this instability, since cleaved Spitz accumulated to higher levels when endocytosis was blocked in a *shibire*^{ts} mutant background in which Drosophila dynamin is inactivated (van der Bliek and Meyerowitz (1991) Nature 351, 411-4.).

This result also indicates that endocytosis is not required for the Rhomboid-1 and Star-induced cleavage of Spitz.

Spitz cleavage in mammalian cells

The proteolytic cleavage assay was recapitulated in a mammalian tissue culture system. Spitz was tagged with GFP near its N-terminus and transiently expressed in COS cells, in the presence or absence of Star and/or Rhomboid-1. The accumulation of the soluble extracellular fragment of GFP-Spitz in the cell medium was then measured by western blot.

Very similar results to those in embryos were obtained: no cleaved Spitz was detected in the absence of Star and Rhomboid-1, nor in the presence of Rhomboid-1 alone. Star induced a low level of cleaved Spitz as well as a new, higher molecular weight full-length Spitz band in the cell lysates. The co-expression of Spitz with Star and Rhomboid-1 led to the efficient cleavage of membrane bound Spitz into a soluble form. Again, Star and Rhomboid-1 were both required for efficient cleavage and they acted synergistically. The size of the released GFP-Spitz fragment was indistinguishable from that of an artificially secreted form of GFP-Spitz, in which the protein was truncated between the EGF domain and the TMD.

The Rhomboid-1 and Star-dependent cleavage of Spitz was not COS cell-specific. Identical cleavage was induced in a broad range of mammalian cell lines, including HeLa, NIH3T3 and CHO.

All cells were tested using the same assay as COS cells i.e. co-transfect Rhomboid, Star and substrate and test medium and lysate for cleaved substrate.

Mammalian cells were also found to be sensitive to over-expression of Rhomboid-1: at the highest levels, Spitz secretion is compromised due to fragmentation of the golgi apparatus when high levels of Rhomboid are expressed.

Spitz is not Cleaved by a TACE-like Metallo-proteases

Spitz, Star and Rhomboid-1 are the only Drosophila proteins present in the transfected mammalian cells. A possible hypothesis is that these proteins are able to recruit a mammalian protease to a processing complex. If this were the case, the principle candidate for the putative mammalian protease would be a member of the ADAM family of metalloproteases. These proteases have broad specificity and are responsible for the release of a large number of mammalian growth factors, including the Spitz homolog, TGFα. The possible involvement of these proteases was tested with the potent metalloprotease inhibitor batimastat (British Biotechnology) at 1μM and 10μM. As expected, batimastat inhibited the release of TGFα at 10μM concentrations in this assay, but it did not affect the cleavage of Spitz by Rhomboid and Star.

The same result was obtained with ilomostat (Calbiochem), another broad spectrum metalloprotease inhibitor. Rhomboid-induced Spitz cleavage is therefore not dependent on a metalloprotease. Interestingly, the lower-level secretion of Spitz induced by Star alone is completely inhibited by 1μM batimastat, implying that this occurs by a mechanism distinct from the Rhomboid-induced cleavage.

Star and Rhomboid-1 are therefore sufficient in themselves to catalyse the cleavage of Spitz.

RNA interference was used to inactivate the Drosophila homologue of TACE (CG7908), the specific metalloprotease required for TGFα cleavage (for which no mutant yet exists in Drosophila). If TACE were necessary for Spitz cleavage, blocking its expression by RNAi should cause a *spitz*-like phenotype. In fact, the embryos thus injected were indistinguishable from wild-type, providing further indication

that Drosophila TACE is not an essential component of Spitz activation.

Intracellular localisation of Spitz, Star and Rhomboid

COS cells expressing combinations of Spitz, Star and Rhomboid-1 were examined to discover the localisation of the proteins. As well as GFP-Spitz, HA-tagged Rhomboid-1 and myc-tagged Star, which were functional in the cleavage assay, were used. Spitz was located only in the endoplasmic reticulum (ER), as demonstrated by its characteristic perinuclear and reticular staining, and by its co-localisation with the ER marker protein disulphide isomerase (PDI). Star had a more complex pattern; it was in the ER, as determined by its co-localisation with PDI. In 80-90% of cells, Star was also in the Golgi apparatus and, in about half of these cells, in the plasma membrane as well. Our finding that Star was present in the ER is consistent with its reported localisation in the Drosophila oocyte (Pickup and Banerjee, 1999 supra). Rhomboid-1 was in the Golgi apparatus, as determined by its co-localisation with the Golgi protein giantin (Seelig et al., 1994 supra); importantly, no Rhomboid-1 could be detected in the ER. Expression of high levels of Rhomboid-1 caused the Golgi apparatus to fragment; in these cells, Rhomboid-1 still co-localised with giantin in the Golgi fragments, but was also seen at the plasma membrane in about 10% of cells. This Golgi fragmentation is a probable explanation for our observation that high levels of Rhomboid reduced Spitz secretion.

Star relocalises Spitz within the Cell

Co-expression of Spitz and Star caused a striking relocalisation of Spitz: instead of being in the ER, Spitz was now located in the Golgi apparatus and the plasma membrane.

When in the Golgi apparatus, Spitz always co-localised with Star, but this co-localisation became less uniform later in the secretory pathway. In some cells, Spitz and Star remained together in the plasma membrane; in others, Spitz was in the plasma membrane whereas Star was confined to the ER and Golgi. These staining patterns suggest that Star and Spitz need to associate for Spitz to move from the ER into the Golgi, but that the subsequent translocation of Spitz through the secretory pathway is not dependent on Star, although the two proteins do sometimes remain co-localised.

The co-expression of Spitz and Rhomboid-1 had no effect on the localisation of either protein: Spitz remained in the ER and Rhomboid in the Golgi apparatus. When Spitz, Star and Rhomboid-1 were co-expressed, Spitz was seen in the Golgi apparatus, providing indication that cleavage and/or subsequent secretion are rate-limiting, not the translocation of Spitz from the ER to the Golgi.

These results indicate that Star regulates EGF receptor signalling by moving Spitz, which is normally retained in the ER, into the Golgi apparatus where it encounters the proteolytic action promoted by Rhomboid-1. In the situation where Spitz and Star are co-expressed in the absence of Rhomboid-1, the uncleaved form of Spitz moves through the Golgi to the plasma membrane. But when the three proteins are co-expressed, Spitz is efficiently cleaved and secreted and therefore does not reach the plasma membrane.

Localisation in Drosophila and yeast cells

HA-tagged Rhomboid was expressed under the control of the Gal4/UAS system in larval salivary glands, which have much larger cells than most in Drosophila and are therefore well-suited for sub-cellular localisation of proteins. All

detectable HA-Rhomboid was located in punctate Golgi-like structures in these cells and it co-localised precisely with a known Golgi marker. This indicates that Rhomboid-1 is indeed localised in the Golgi apparatus in Drosophila, and that it does not normally reside in the plasma membrane. This localisation is evolutionarily conserved, as tagged versions of the *Saccharomyces cerevisiae* Rhomboid homologue YPL246c was also localised in intracellular Golgi-like compartments and were not associated with the plasma membrane. The *Saccharomyces cerevisiae* Rhomboid homologue YGR101w was found to be localised in the mitochondria.

A biochemical approach was used to analyse the location of Spitz in Drosophila cells, and to determine whether it is relocalised by Star. Spitz protein migrated at a higher molecular weight than expected on western blots, and the bands had a diffuse appearance. This provides indication that, as predicted, and like TGFα (Teixido et al., (1990) J Biol Chem *265*, 6410-5.; Rutledge et al., (1992) Genes Dev. *6*, 1503-1517; Schweitzer et al., 1995 supra), the extracellular portion of the molecule is glycosylated.

This was confirmed with a variety of deglycosylation enzymes, and the specificity of these enzymes allowed us to infer the sub-cellular localisation of Spitz. Endo-H removed all evidence of glycosylation from the form of full-length Spitz that occurs in the absence of Star. As Endo-H is an enzyme that removes only simple, high-mannose N-linked glycosylation characteristic of ER modification, this indicates that this form of Spitz is resident only in the ER.

As previously noted, when Spitz was co-expressed with Star, a band of increased molecular weight appeared; Endo-H could not deglycosylate this form of Spitz. Instead, it was sensitive to enzymes that remove O-linked sugars that are added only in the

Golgi apparatus (O-glycosidase and neuraminidase). The cleaved form of Spitz had the same pattern of sensitivity as the Star-dependent form. These results show that in Drosophila embryos, Spitz is located solely in the ER until Star exports it to the Golgi apparatus, where it acquires O-glycosylation. Rhomboid did not affect the glycosylation of Spitz: in embryos, as in mammalian cells, its only function seems to be to promote cleavage.

Essential Domains of Spitz

A series of GFP-tagged chimeras between Spitz and human TGFα and deletions based on those of Bang and Kintner (Bang and Kintner (2000)supra) were examined in the COS assay (Fig. 1). TGFα was constitutively secreted from COS cells efficiently by a mechanism that was sensitive to metalloprotease inhibitors; the addition of Star and/or Rhomboid had no detectable effect either on its secretion or its localisation throughout the secretory pathway and at the plasma membrane.

Requirement for Star-dependent relocalisation

The replacement of the TGFα TMD with that from Spitz did not affect its broad distribution. In contrast, TGFα chimeras containing the Spitz cytoplasmic domain were retained tightly in the ER, implying that this domain contains the information necessary for Spitz ER retention. Deletion, however, of the 53 C-terminal amino acids of Spitz (leaving only the 13 membrane-proximal amino acids of the cytoplasmic domain) did not compromise ER retention.

The property of being relocalised by Star did not map to a single domain of Spitz. Deletion of the 15 residues between the EGF domain and the TMD reduced the efficiency of relocalisation substantially: some was relocalised to the Golgi apparatus by Star but much remained in the ER ; the poor relocalisation of Spi-Δ15 was confirmed by the absence of a

shifted, O-glycosylated full-length band in the cell lysates. In contrast, removal of only the 8 juxtamembrane residues did not reduce Star-dependent relocalisation. Removal of the cytoplasmic C-terminal 53 residues also made Star relocalisation less efficient than wild-type – again, some Spitz was retained in the ER; in this case a weak O-glycosylated band is visible in cell lysates, indicating that more of Spi-Δ53C than Spi-Δ15 gets to the Golgi.

Together, these results show that both the lumenal and cytoplasmic domains of Spitz are involved in relocalisation. However, another chimera demonstrated that the lumenal domain of Spitz is sufficient for Star-dependent relocalisation. Thus, a construct which comprises the extracellular domain of Spitz linked to the TMD and cytoplasmic domain of TGFα had a distribution indistinguishable from TGFα (ER, Golgi, some plasma membrane) but upon co-expression of Star, it was no longer detectable in the ER and the cell surface staining became more prominent. Therefore, despite the absence of the Spitz ER retention signal, this chimera was re-localised by Star. In contrast, TGFα chimeras containing the Spitz cytoplasmic domain were not re-localised by Star, indicating that this domain is not sufficient for Star re-localisation.

Requirement for Rhomboid-dependent cleavage

Strikingly, the replacement of the TGFαTMD (trans-membrane domain) with the Spitz-TMD was sufficient to render the chimera (TGFα:SpiTM) sensitive to Rhomboid-1-promoted cleavage, albeit with variable efficiency. This cleavage was apparent in the cell lysates as new Rhomboid-1-dependent bands; it was not detectable in the medium, although this may be due to poor secretion of the cleaved product or it may simply have been obscured on the gel by the high level of

constitutive secretion of this chimera. Consistent with the TMD being the main determinant of Rhomboid-1-dependent cleavage, a chimera containing the Spitz extracellular domain with the TGFα TMD and cytoplasmic domain was not cleaved by Rhomboid-1; in the presence of Star, the O-glycosylated form of the full length protein accumulated in the cells. The converse TGFα:Spi-TMC chimera was also not detectably cleaved by Rhomboid.

Although this result appears at odds with the sufficiency of the Spitz TMD to confer Rhomboid sensitivity, the localisation of the TGFα:SpiTM and TGFα:Spi-TMC chimeras were quite distinct: the former was distributed throughout the secretory pathway, while the latter was tightly ER localised by virtue of the Spitz cytoplasmic domain. TGFα:Spi-TMC was therefore not exposed to Rhomboid-1 in the Golgi apparatus, explaining why no cleavage was detected.

Consistent with the idea that the TMD of Spitz confers Rhomboid-1 sensitivity, Spi:TGFαTMC is not cleaved by Rhomboid-1 whereas Spi-Δ53C is. The significance of the TMD is challenged, however, by published observations that deletion of the 15 amino acids between the extracellular face of the membrane and the EGF domain (Spi-Δ15) caused an apparent failure of Spitz cleavage (Bang and Kintner, 2000 supra). This construct activated EGF receptor signalling in a Xenopus explant assay in a Rhomboid and Star-dependent manner and it was concluded in this paper that the cleavage of Spitz was not the primary function of Rhomboid and Star.

However, in our assay Spi-Δ15 is cleaved in a Rhomboid-1 and Star-dependent manner, albeit at reduced efficiency, implying that the 15 residues between the membrane and EGF domain are not essential for Rhomboid-induced cleavage. Indeed, the reduced efficiency of cleavage of this construct could be

entirely due to its diminished ability to be re-localised by Star. This result explains the discrepancy between our results and those presented previously (Bang and Kintner (2000) supra).

In summary, all the present results are consistent with the TMD of Spitz being necessary and sufficient for Rhomboid-1-dependent cleavage.

Rhomboid-1 Activity does not require Star

A prediction of our model is that a form of Spitz that was not retained in the ER would be cleaved by Rhomboid-1 in the absence of Star. To test this prediction, another Spitz:TGFα chimera was made, this time comprising Spitz with only its cytoplasmic domain replaced by the TGFα cytoplasmic domain (Spi:TGFα-C). The localisation of this construct resembled TGFα, although more of it was retained in the ER. In about 20% of cells it was visible in the Golgi apparatus and occasionally at the cell surface. This was never seen with Spitz, which is always ER-localised. Since Spi:TGFα-C has the Spitz lumenal domain, it was moved out of the ER by Star. Importantly, it was efficiently cleaved by Rhomboid-1 even in the absence of Star. The addition of Star did enhance Rhomboid-1 dependent cleavage, consistent with Star's ability to chaperone Spi:TGFα-C out of the ER.

This result demonstrates that the function of Star is to relocalise Spitz; it also demonstrates that Rhomboid-1-dependent cleavage does not require Star as a cofactor. Since Spi:TGFα-C is cleaved by Rhomboid whereas Spi:TGFα-TMC is not, and the only difference between them is their TMDs, this result provides indication that the Spitz TMD confers Rhomboid-1 sensitivity. These results were confirmed by the

use of KDEL tagged Rhomboid which is localised in the ER as described below.

The Lumenal Domain of Star is required for its Function

The lumenal domain of Spitz is sufficient to confer sensitivity to Star-dependent relocalisation so we tested whether the lumenal domain of Star was essential for its relocalising function. Three C-terminal truncations were tested which removed 291, 266 and 47 lumenal amino acids respectively (Star is a type 2 protein), and all abolished the ability of Star to relocalise Spitz significantly from the ER to the Golgi apparatus.

Consistent with this, these truncations were unable to mediate Star-dependent glycosylation of Spitz, or to induce Rhomboid-1-dependent cleavage. All three truncations were expressed at normal levels and with the same intracellular localisation as wild-type Star. Therefore, the lumenal domain of Star is necessary for its ability to relocalise Spitz, consistent with the idea that the primary interaction between Spitz and Star is lumenal.

The TMDs contain the Core Function of Rhomboid-1

All members of the Rhomboid family have an N-terminal hydrophilic domain followed by the region containing the TMDs. In the case of Rhomboid-1, we have determined that the N-terminal domain is cytoplasmic. The lack of signal peptides in other Rhomboids provides indication that this topology is conserved. Despite the ubiquity of these N-terminal domains, they have no detectable sequence conservation, so their function is unclear.

The ability of the N-terminal alone (Rhomboid-1N) or the transmembrane domains without the N-terminal (Rhomboid-1ΔN) to

promote Spitz cleavage was tested by expressing Rhomboid-1N and Rhomboid-1ΔN in the presence of substrate (GFP-Spitz) and Star in COS cells and assaying cleavage of substrate. The soluble cytoplasmic N-terminal had no activity, whereas Rhomboid-1ΔN cleaved Spitz, albeit with reduced activity.

Importantly, the Rhomboid-1ΔN cleavage was insensitive to 1μM and 10μM batimastat, confirming that the cleavage was not an artefact caused by metalloprotease-dependent cell surface shedding.

Identical results were obtained in vivo when these constructs were expressed in wings using the GAL4/UAS system (Brand and Perrimon (1993) Development 118 401-415): Rhomboid-1ΔN has similar activity to full-length Rhomboid-1 whereas Rhomboid-1N has no activity. To examine this further we made an extensive series of N and C-terminal truncations of HA-tagged Rhomboid-1 and tested their ability to promote Spitz cleavage in a standard COS cell assay. The only one of this series to retain some (reduced) activity was N8, in which the C-terminal lumenal domain of Rhomboid-1 was deleted. All others removed at least one TMD and all activity was lost (see Table 3 and figure 4).

Together, these results demonstrate that the core function of Rhomboid-1 – its ability to promote Spitz cleavage – resides in the part of the protein with the multiple TMDs, not in the cytoplasmic N-terminus nor the lumenal C-terminus.

Rhomboid is a Proteolytic Enzyme

A key feature of enzymes is that they act catalytically in a reaction, at sub-stoichiometric levels. Conversely, reaction substrates and proteins with non-catalytic roles in a reaction behave stoichiometrically – reducing the amount of such

proteins is expected to produce a proportional reduction in reaction products. Rhomboid-1 was tested in accordance with this principle for enzymatic behaviour in promoting the cleavage of Spitz.

The concentration of each of the three components of the cleavage reaction was titrated by reducing the amount of DNA transfected into COS cells. The total DNA was maintained at 1µg per transfection, to ensure that the transfection efficiency remained constant. The amount of cleaved GFP-Spitz in the medium after 24-30 hours was determined by western blot.

Decreasing the amount of Spitz in the reaction was observed to have a linear effect on the amount of product. This is as expected, given that Spitz is the substrate for this reaction.

Reduction of Star was observed to have a strong effect on the overall efficiency of the Spitz cleavage reaction: the less Star is present, the less Spitz can be relocalised. This is consistent with the mechanism of Star acting as a chaperone protein that functions by interacting with Spitz and translocating it to the Golgi apparatus.

In contrast, reducing rhomboid-1 DNA by 10-fold and 100-fold actually increased its cleavage ability (by reducing Rhomboid-1's Golgi-disrupting effect). At 10,000-fold dilution (i.e. 0.025 ng of rhomboid-1 DNA per transfection), Rhomboid-1 is still effective at promoting Spitz cleavage.

An important control for this experiment was the demonstration that the amount of DNA transfected into the cells was directly proportional to the level of protein expressed (i.e. that HA-tagged Rhomboid-1 protein levels diminished in proportion to the input DNA). This was tested by probing western blots of cell lysates with anti-HA antibody to measure directly HA-

Rhomboid-1 protein levels. Even at the first dilution in the series (10-fold), the level of Rhomboid-1 was reduced to below detectable. This confirms that Rhomboid-1 efficiently promotes proteolytic activity at extremely low levels. The contrast between this property of Rhomboid-1 and the sensitivity of Star and Spitz to reduction in their levels, demonstrates that Rhomboid functions sub-stoichiometrically, the hallmark of an enzyme.

Similar titration experiments were performed in other cell lines (CHO, NIH3T3 and HeLa) and the same results were obtained, implying that the sub-stoichiometric function of Rhomboid-1 is a general property of this protein.

If Rhomboid-1 was acting as an enzyme, certain residues within the Rhomboid 1 sequence would be expected to form the catalytic site. The activity of an enzyme would be especially sensitive to mutagenesis of residues in this catalytic site: in fact their alteration should completely abolish enzymatic activity, if they are genuinely part of the catalytic function.

In contrast, residues that are involved with other aspects of Rhomboid-1 function (e.g. binding to other proteins, protein conformation) may be less sensitive to alteration.

All residues that are highly conserved within the Rhomboid family (excepting a few glycines, which would be predicted not to have a catalytic function) were individually mutated in Rhomboid-1. The ability of these mutated forms of Rhomboid-1 to promote Spitz cleavage was then tested in the COS cell assay by western blot analysis of cleaved GFP-Spitz in the medium.

The expression of the mutant forms (which were all HA-tagged) was confirmed by probing cell lysates with anti-HA antibody:

all were expressed at comparable levels. Of the 13 highly conserved residues, only 6 were essential for Rhomboid-1 function. Alteration of any one of these six to alanine (W151, R152, N169, G215, S217, H281) completely abolished Rhomboid-1 activity. Given the sensitivity of this assay (even a 1:1000 dilution of the normal input amount of rhomboid-1-HA DNA had detectable activity), it can be concluded that each of these single mutations reduced the proteolytic function by at least 1000-fold.

Mutation of other conserved residues led to a detectable but incomplete reduction in Rhomboid-1 function (R188, G218) or activity indistinguishable from wild-type function (S155, H160, H165, E181, Y193). Note that the assay is so sensitive to Rhomboid function that these latter cases might reduce activity significantly without leading to a detectable reduction in cleaved Spitz in the medium. Therefore, despite their activity in this assay, these residues may be important for some aspect of Rhomboid-1 function, but they cannot be essential for catalysis itself.

Rhomboid-1 is a Serine Protease

Rhomboid-1 has been shown to possess the properties of a protease that cleaves Spitz. However, since there are no well-conserved cysteine or aspartic acid residues in the Rhomboid proteins, Rhomboid-1 is unlikely to be a cysteine or aspartyl protease. The other two classes of known proteases are metalloproteases and serine/threonine proteases.

Rhomboid-1 has a number of conserved histidine residues that could act to coordinate a metal ion, which might indicate that it is a metalloprotease. However, the mutagenesis analysis described above indicates that most of these residues are not part of the catalytic mechanism. Furthermore, Rhomboid-1-dependent cleavage of Spitz has been found to be insensitive

to the potent inhibitors of metalloproteases, batimastat and ilomostat. The batimastat assay was performed over a range of Rhomboid-1 concentrations (2.5, 0.25 and 0.025ng Rhomboid DNA per 35mm well of transfected COS cells in 1µM and 10µM Batimastat) to search for any batimastat sensitivity, even at concentrations where Rhomboid-1 is limiting.

Under all conditions tested, batimastat at 1µM and 10µM had no effect on Rhomboid-1-dependent cleavage, confirming the conclusion that the cleavage of Spitz is not catalysed by a metalloprotease.

The site directed mutagenesis described above focussed our attention on a cluster of residues in TMD4 of Rhomboid-1. In the GASGG motif, the first glycine (G215) and the serine (S217) are both essential for catalytic activity. This sequence is conserved in almost all Rhomboid homologues that exist in sequence databases. Strikingly, it is similar to the GXSGG sequence in many serine proteases including chymotrypsin and trypsin, where the serine is the active residue in the hydrolytic reaction itself. In these serine proteases, the first glycine in this motif also has an important function (though it is not a component of the catalytic triad), hydrogen bonding to the peptide backbone of the substrate. The serine protease catalytic triad also includes a histidine. In Rhomboid-1 histidine 281, in TMD6, is one of the other essential residues and it is predicted to be in a similar position in the lipid bilayer as the putative catalytic serine. They could therefore form part of a serine protease active site. Such a serine protease active site would be entirely novel, as it occurs within a lipid bilayer.

The third residue of the serine protease catalytic triad is an aspartate; there are no conserved essential aspartates in Rhomboid-1.

Interestingly, asparagine-169 in TMD2 of Rhomboid-1 is essential; it is predicted to reside at a similar level in the bilayer as serine-217 and histidine-281 and therefore is a candidate to be involved in Rhomboid-1 catalysed proteolysis. A hydrophobic cysteine protease is known in which the aspartate of the catalytic triad is replaced with an asparagine (Vernet et al (1995) J. Biol. Chem. 270 16645-16652). Cysteine proteases have very similar catalytic mechanisms to serine proteases, so this provides indication that N-169 substitutes for the aspartate in the Rhomboid catalytic triad.

The function of the last two essential residues (W151 and R152) has not been established. They are predicted to be in the lumenal loop between TMD1 and TMD2.

The experiments described herein demonstrate that Rhomboid-1 is a novel serine protease that cleaves substrates within their transmembrane domains and that R152, G215, S217 and H281 are the key catalytic residues, forming the catalytic centre and/or essential docking sites, with W151 and N169 also being of some importance.

This is strongly supported by our observation that Rhomboid-1-dependent cleavage of Spitz was not inhibited by specific inhibitors of metalloproteases but was sensitive to serine protease inhibitors. We performed the standard cleavage assay in the presence of a panel of inhibitors.

No inhibition of Rhomboid activity was observed with the following inhibitors; cysteine protease inhibitors E64d (50μM) and leupeptin (100μM) (Salvesen, G. S., and Nagase, H. (2001). Inhibition of proteolytic enzymes. In Proteolytic Enzymes, R. Beynon, and J. S. Bond, eds. (Oxford, Oxford University Press), calpain inhibitor PD150606 (Wang, K. K.*et al.* (1996). Proc Natl Acad Sci U S A 93,6687-6692.), aspartyl

protease inhibitor pepstatin A (50μM) (Salvesen, G. S., and Nagase, H. (2001). Inhibition of proteolytic enzymes. In Proteolytic Enzymes, R. Beynon, and J. S. Bond, eds. (Oxford, Oxford University Press), gamma secretase inhibitor I (25μM) (Hartmann, T. et al(1997). Nat Med 3, 1016-1020), and metalloprotease inhibitors batimastat (British Biotech) and ilomostat (Calbiochem).

However, two serine protease inhibitors (TPCK and 3,4-DCI) were observed to strongly inhibited the reaction (10-100μM). Importantly, this concentration of DCI and TPCK did not effect the expression or secretion of an artificially truncated form of Spitz (the extracellular domain, missing the transmembrane and cytoplasmic domain). This indicates that DCI and TPCK affect Spitz cleavage itself, not its expression or secretion. This provides direct evidence that Rhomboid-1 is a novel serine protease. This being so, it is the first described serine protease in which the catalytic site occurs within the lipid bilayer of a membrane.

Human Rhomboid Homologues

The programs tblastn and blastp were used to search public sequence databases for Rhomboid genes. The following three human sequences, which had greater than 40% similarity to the Drosophila rhomboids, were identified (with GenBank accession numbers);

1) XM_007948, NM_003961, AJ272344 (different numbers represent different submissions of the same gene); this corresponds to the RHBDL gene identified and published by Pascall and Brown (FEBS Lett. 429, 337-340, 1998).

2) NM_017821; this gene was identified in the human genome project as a predicted gene and full-length cDNAs have been isolated. Although it was annotated as having similarity to

Drosophila rhomboid, it has otherwise not been named or characterised. The name RHBDL2 has been officially accepted by the human gene nomenclature committee.

3) BE778475; this is only an incomplete cDNA; the gene was not identified or annotated in the human genome project. Nor was the sequence annotated as having any rhomboid similarity. Using the Genemark program (Borodovsky M. and McIninch J. Computers and Chemistry (1993) 17 19 123-133) we searched the surrounding genomic DNA sequence to identify the full length sequence. This full length sequence is shown in Figure 7.

The full-length gene sequence shows significant similarity with RHBDL-1 and RHBDL2 over its entire length (52.6% identity, 60.2% similarity to RHBDL1; 35.0% identity, 47.7% similarity to RHBDL2; 34.7% identity, 45.7% similarity to Drosophila rhomboid-1). The name RHBDL-3 has been officially accepted for this gene. Importantly, the RHBDL3 gene product contains all the conserved residues shown to be catalytically essential for Rhomboid protease function. The gene therefore encodes a true Rhomboid with proteolytic activity.

Substrate Specificity of Drosophila Rhomboids

The ability of three other Drosophila Rhomboids to cleave Spitz and two other Drosophila TGFα-like ligands, Keren and Gurken was assessed.

Drosophila Rhomboid-1, -2, -3 or -4 were expressed in COS cells and their ability to cleave the ligands was compared over a range of Rhomboid expression levels (from 25ng to 0.05ng per transfection). Cleavage was assayed by western blotting of the 24 hour-conditioned medium for the presence of soluble GFP-Spitz. All four Rhomboids cleaved Spitz efficiently using the GAL4/UAS system (Brand & Perrimon supra). We have confirmed this result in vivo: the ectopic

expression of all four Rhomboids leads to similar phenotypes typical of EGF receptor hyperactivity, such as rough eyes and extra wing vein material. It can therefore be concluded that the proteolytic activity of Rhomboid-1 has been conserved in all four of the Drosophila Rhomboids tested. Note that in all cases, the residues identified herein as being essential are conserved, as is the overall predicted structure of the protein, but otherwise there is quite wide divergence of sequence homology, especially between Rhomboids 1 and 4.

The ability of Drosophila Rhomboids 1 to 4 to promote the cleavage of two other TGFα-like Drosophila ligands, Gurken and Keren was also analysed in a COS cell cleavage assay and in a similar assay performed in Drosophila S2 tissue culture cells. Gurken was efficiently cleaved by all four Rhomboids tested but unlike Spitz, the cleavage efficiency was independent of Star. All four Rhomboids fully cleaved the full length form of Gurken but Rhomboid-1 consistently produced two intracellular cleaved products, whereas Rhomboids 2 to 4 produced only one intracellular band. The fact that all four Rhomboids cleave Gurken supports the earlier conclusion that they all have the same core proteolytic activity, but the observation that Rhomboid-1 causes a different pattern of cleavage indicates that their action is not identical.

This specificity is further highlighted in the case of Keren cleavage. Again, all four Rhomboids promoted cleavage, but in this case, the differences were more pronounced.

In the absence of Star, Rhomboids 1 and 2 led to inefficient cleavage of Keren; the level of cleavage was so low that it could only be detected as a minor novel band in the cell lysate (unlike Gurken, most of the detectable Keren remained uncleaved) and it did not accumulate to detectable levels in the medium.

In contrast, Rhomboids 3 and 4 catalysed more efficient Star-independent cleavage and secretion of Keren; the cleaved product was visible in the cell lysate and also accumulated substantially in the medium.

In the presence of Star, the overall efficiency of cleavage and secretion of Keren was enhanced but the differential between Rhomboids 1 and 2 on one hand and Rhomboids 3 and 4 on the other, was maintained, as assayed by the intensity of the cleaved band in the cell lysates.

Interestingly, as with Gurken, Rhomboid-1 cleavage produced two products in the cell lysates, while Rhomboids 2 to 4 produced a single band. On the basis of these results we conclude that Rhomboids 3 and 4 are much more efficient than Rhomboids 1 and 2 at cleaving Keren; and that Rhomboid-1 has a distinct cleavage action, apparently cleaving Keren at two sites. Again, these results indicate that there is some substrate specificity between different Rhomboids.

Proteases often display specificity for their substrates to achieve the precision required to regulate biological processes (as reviewed in Perona, J. J., and Craik, C. S. (1997). *J Bioi Chem,* 272: 29987-90). Despite cleaving Spitz, Gurken and Keren, Rhomboid-1 could not cleave other type-I membrane proteins including *Drosophila* EGF receptor, Drosophila Delta, human TGN38 (Luzio, J. P ., Brake, B., Banting, G., Howell, K. E., Braghetta, P., and Stanley, K. K. (1990) *Biochem J,* 270: 97-102) and human TGFα.

Furthermore, analysis of Spitz cleavage in the ER by Rhomboid-1-KDEL provides indication that Rhomboid-1 alone is responsible for this specificity. KDEL is an ER-retention signal that retains the KDEL-tagged Rhomboid-1 polypeptide in the ER. Cleavage by Rhomboid-1-KDEL does not rely on Spitz

trafficking by Star, and this allows a direct test of whether Star has a secondary role in substrate presentation to Rhomboid-1.

Although it has been proposed that Star physically binds to both the Spitz substrate and the Rhomboid-1 protease (Hsiung, F., Griffis, E. R., Pickup, A., Powers, M. A., and Moses, K. (2001) *Mech Dev,* 107: 13-23) (Tsruya, R., Schlesinger, A., Reich, A., Gab ay, L., Sapir, A., and Shilo, B. Z. (2002). *Genes Dev,16:* 222-34.), the amount of intracellular cleavage catalysed by Rhomboid1-KDEL was not observed to be enhanced when Star was co-expressed with various forms of Spitz. Therefore the specificity and proteolytic activity of Rhomboid-1 is fully independent of Star.

These observations indicate that Rhomboid-1 is highly selective in its choice of substrate and demonstrate that the specificity in Spitz cleavage is determined by Rhomboid-1 alone.

Cleavage of Spitz by Human RHBDL2

A full-length cDNA for Human RHBDL2 was listed in the Japanese NEDO human cDNA sequencing project (clone number FLJ20435).

The Human RHBDL2 cDNA was expressed in COS cells and its ability to induce Spitz cleavage in the presence of Star was analysed. GFP-Spitz, Star and Human RHBDL2 were expressed in COS cells. Accumulation of GFP-Spitz in the medium was assayed in the presence of 10μM batimastat (to inhibit background metalloprotease activity). Under these conditions, human RHBDL2 efficiently catalysed GFP-Spitz cleavage.

This provides further demonstration that the core proteolytic function of Rhomboid is conserved between Drosophila and

humans, as is further evidenced by the conservation of the key catalytic residues. This conservation of sequences between Drosophila Rhomboid-1 and RHBDL2 (all the key catalytic residues are identical) provides further indication that the these proteases work by the same mechanism.

The six residues found to be important for Rhomboid-1 activity were mutated in human RHBDL2. Mutation of R111, G174, S176 or H239 (numbered according to the human RHBDL2 sequence) to alanine completely abolished the proteolytic cleavage of Spitz by human RHBDL2. Mutation of W110 and N128 reduced, but did not abolish proteolytic activity. The conservation of key residues in RHBDL2 demonstrates that the proteolytic cleavage of Spitz by RHBDL2 occurs through the same mechanism as proteolytic cleavage by Rhomboid-1.

The catalytic serine (typically GASG, although variants at positions 2 and 4 exist) motif is shown herein to be conserved as the catalytic centre of an intramembrane protease in members of the family well separated in evolution (Rhomboid-1, human RHBDL2, bacterial and yeast RHBDLs). The provides indication that the same enzymatic activity is conserved across the whole family of conserved Rhomboid-like proteins, which are shown to be related intramembrane proteases.

Truncated Forms of Rhomboid-1 act as Dominant Negatives

A truncated form of Rhomboid-1 was made comprising residues 1 to 149 of Rhomboid-1, the N-terminal cytoplasmic domain plus the first TMD and part of the first extracellular loop (Rho-1-NTM1).

When co-expressed with Star and full-length Rhomboid-1 in the COS cell Spitz cleavage assay, the truncated Rhomboid-1 inhibited the ability of the wild-type protein to promote GFP-

Spitz cleavage. There are two obvious explanations for this result: either the truncated Rhomboid is acting as a dominant negative construct (i.e. it is specifically interfering with the Rhomboid-1 cleavage event) or it might be non-specifically disrupting the cells' viability or ability to secrete proteins.

Rho-1-NTM1 did not interfere with TGFα synthesis or secretion, which was indistinguishable from a control. Therefore, this N-terminal fragment of Rhomboid-1 has the ability to inhibit Rhomboid-1-dependent Spitz cleavage quite specifically, providing indication that it is indeed a dominant negative form of the protein.

The dominant negative activity of this Rhomboid-1 fragment was confirmed in vivo by expressing it in Drosophila using P-element mediated transformation. When expressed in the developing eye using the Gal4/UAS system (Brand and Perrimon), it caused cell death, a phenotype associated with loss of Rhomboid-1 and Rhomboid-3 function.

Since the overall structure and topology of proteins of the Rhomboid family is well conserved, and since the catalytic mechanism appears to be conserved (four distinct Drosophila Rhomboids have the same core catalytic activity and the potential catalytic residues we have identified are conserved throughout most of the family), it follows that the dominant negative activity of similar N-terminal fragments of other Rhomboids may be employed. This may provide techniques for determining the role of Rhomboids from any species, regardless of whether mutations in the genes exist, as well as for manipulating Rhomboid activity for practical purposes.

Genetic analysis has implied that Star and Rhomboid-1 are the primary regulators of Drosophila EGF receptor activation but their mechanisms have remained elusive until now (reviewed in

Schweitzer and Shilo, (1997) Trends in Genetics *13*, 191-196; Wasserman and Freeman, (1997) Cell *95*, 355-364; Klämbt, (2000) Curr Biol *10*, R388-91). We have now determined the mechanism of each of these molecules: Star is necessary for the export of the activating ligand Spitz from the ER to the Golgi apparatus. There, Spitz encounters Rhomboid-1, which is a transmembrane serine protease which cleaves the ligand.

Cleavage itself is a new variation of regulated intramembrane proteolysis (Brown et al. (2000) Cell *100*, 391-8) which occurs within the Spitz TMD. Once cleaved, the soluble lumenal ligand fragment is secreted from the cell to trigger the activation of the EGF receptor. Thus the rate limiting steps of the Drosophila EGF receptor signalling pathway occur primarily at the level of ligand translocation and proteolytic cleavage.

Spitz Recognition and Cleavage

To determine the location of Spitz cleavage, the size of the Rhomboid-1-cleaved product was compared with the size of Spitz fragments expressed from artificially truncated open reading frames. The cleaved product was detectably larger than a form of Spitz truncated immediately N-terminal to the TMD (residue 139), but smaller than a truncation at residue 149, which is 2/3rds into the TMD (from the lumenal surface). Therefore Spitz is cleaved between residues 139 and 149. Since the resolution of this assay is approximately 5 residues, this places the cleavage site at approximately residue 144, within the lumenal half of the TMD. This is the same 'height' within the TMD as the proposed Rhomboid-1 active site. No other intramembrane protease is known to cut TMDs towards their lumenal/extracellular side.

Spitz TMD was sequentially replaced with that of TGFα in four nested segments starting from the cytoplasmic end (see Figure

6). This was done in a chimeric Spitz molecule that had the TGFα C-terminus to confer a Star-independent Golgi localisation. Replacing the bottom 1/4 (Spitz residues 155-160), 2/4 (residues 150-160), or 3/4 (residues 145-160) of the Spitz TMD with that of TGFα did not affect cleavage by Rhomboid-1. However, replacing the remaining 5 residues (residues 140-160) abolished cleavage. Star was not used in these assays, and they were done in the presence of batimastat to remove background cleavage by cell-surface metalloproteases.

To exclude the possibility that replacing the entire Spitz TMD with that of TGFα resulted in masking of the Rhomboid-1 recognition site by TGFα-specific binding proteins rather than by loss of the recognition site, a similar analysis with a different TMD was performed: this time the Spitz TMD was sequentially replaced with segments from an unrelated TMD — from the mammalian protein TGN38. This yielded identical results, confirming that the five TMD residues of Spitz closest to the lumenal face contain the site that Rhomboid-1 recognises.

This analysis pinpointed Spitz residues 140-144 (IASGA) as containing the principal Rhomboid-1 recognition site; when replaced by the equivalent sequence from TGFα (ITALV), this abolished cleavage by Rhomboid. In a primary analysis, each of these was mutated individually in wild-type Spitz. Only the A141T, G143L, and A144V mutations reduced Rhomboid-1 cleavage, each doing so by about 3-5 fold. The G143L mutation conferred the strongest effect, while the S142A mutation had no detectable effect on Spitz cleavage.

Computer predictions of the Spitz TMD (e.g. with the program TMHMM - Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer,

E.L. (2001). *J Mol Bioi*, 305, 567-580.) indicate that the TMD actually extends two residues N-terminal of the sequence replaced in the Spitz/TGFα chimera series described above. Therefore, in conjunction with the results of the TGFα chimeras, the residues ASIASGA (residues 138-144) were analysed for their role in determining Spitz cleavage.

First, in order to test which of the residues in the critical region of Spitz were responsible for recognition by Rhomboid-1, each of the residues found in Spitz was introduced individually into the TGFα TMD of the uncleavable Spitz-TGFα chimera (i.e. Spitz with residues 140-160 replaced by the equivalent residues from TGFα). Remarkably, substitution of only Spitz residue G143 restored cleavage of this chimeric substrate, while substitution of both G143 and A144 resulted in more efficient cleavage. Thus, Rhomboid-1 appears to target this GA motif within Spitz. Intriguingly, the exact position of this GA motif within the substrate TMD is not absolutely constrained as Rhomboid-1 could cleave Spitz with the GA motif displaced one residue and three residues further into the TMD.

Second, in a complementary approach similar to the primary analysis described above, all of the critical region residues were mutated singly in wild-type Spitz. The ability of these mutant forms of Spitz to act as substrates for Rhomboid-1 was assessed using Rhomboid-1-KDEL (which allows cleavage in the ER to be assayed and avoids variation of secretion efficiency causing misleading results). This is significant because since many of the mutated forms of Spitz differ in their ability to be secreted. Under these conditions, the amount of cleavage was dependent on the quantity of Rhomboid-1-KDEL in cells, and it was possible to use a concentration of Rhomboid-1-KDEL that resulted in approximately 50% cleavage such that mutations that both enhanced and suppressed cleavage could be

identified. The results are summarised in Fig 9, where the wild-type sequence is shown along the top of the table and the mutations tested are shown below their respective wild-type residues. Asterisked mutations abolished Spitz cleavage; unlabelled mutations had little or no effect; and those underlined enhanced cleavage. The vertical lines show the limits of the experimentally-determined critical region.

This analysis of Spitz mutants revealed two further characteristics of the critical region. First, although the first three positions could not accommodate large, disruptive residues such as phenylalanine, the presence of such residues was less important in the second half of the critical region. Consistent with the restoration analysis, G143F was the only residue of the second half of the critical region to be sensitive to phenylalanine mutagenesis. These results suggest that, in addition to G143 , the residues of the top half of the critical region may have a role in recognition by Rhomboid-1. Interestingly, the disruptive nature of the A138F mutation was likely to be due to increasing hydrophobicity rather than size at this site since a tyrosine substitution was cleaved efficiently. Thus, the first few residues of the Spitz TMD may need to be limited in hydrophobicity, perhaps to allow water to pass into the Rhomboid active site. Secondly, four mutations were isolated that enhanced cleavage by Rhomboid-1, and these were residues that are generally thought to destabilise helices. Two of these residues were very small; mutation of the A144 of the GA motif to the smaller residue glycine and mutation of S139 to the smaller alanine residue both enhanced cleavage. Mutation of residues 141 and 143 to β-branched residues threonine and isoleucine, respectively, also enhanced cleavage. Thus, certain small and β-branched residues were effective for enhancing cleavage by Rhomboid-1.

Collectively, these results indicate that Rhomboid-1 does not recognise a specific sequence, but rather a structural determinant, apparently a common disordered conformation. Small residues such as those of the GA motif are not as constrained in their conformation as larger residues, and are thus known to destabilise rigid structures such as helices (Chou, P. Y., and Fasman, G. D. (1978). *Annu Rev Biochem,* 47: 251-76. Parker, M. H., and Hefford, M. A. (1997) *Protein Eng,* 10: 487-96. Liu, L. P., and Deber, C. M. (1998) *Biopolymers,* 47: 41-62. Butcher, D. J., Luo, Z., and Huang, Z. (1999). *Biochem Biophys Res Commun,265:* 350-5.). Furthermore, β-branched residues also have lower propensity to be in helical structures. Thus, the critical region of the Spitz TMD may be able to adopt a non-helical conformation, in contrast to many TMDs.

Drosophila Rhomboids 2, 3,4 and human RHBDL-2 were tested for protease activity against the uncleavable form of Spitz (in which residues 140-160 replaced by the TGFα TMD), and against the TGFα TMD with a restored G143. Rhomboids 2,3, and RHBDL behaved exactly as Rhomboid-1: they could not cleave the TGFα TMD, but did cleave when only G143 was added back. This indicates that these Rhomboids all use the same mechanism to recognise polypeptide substrate.

Rhomboid-4 Specificity

Drosophila Rhomboid-4 is distinct from the other Rhomboid polypeptides that have been tested (e.g. Drosophila Rhomboids 1,2,3 and human RHBDL2). Whilst many Rhomboid polypeptides have defined and restricted specificities for substrate recognition, Rhomboid-4 cleaves all the polypeptide substrates tested so far, including Spitz, Gurken, Keren, human TGFα, the 'non-cleavable' Spitz/TGFa chimera, and the completely unrelated Drosophila EGF receptor.

Therefore, unlike the other Rhomboid polypeptides, Rhomboid-4 is a broad spectrum intra-membrane protease which is capable of cleavage of the TMD of a wide range of proteins.

Bacterial Rhomboids

Although the Rhomboid family does not possess high overall homology, bioinformatic tools as described herein, such as PFAM and psi-Blast, may be used to identify family members in all branches of evolution.

Table 1 provides examples of members of the Rhomboid family identified in this way. Intra-membrane protease activity similar to that of Drosophila and human Rhomboid polypeptides, has been also observed for other members of the Rhomboid family.

Rhomboid polypeptides have been cloned from the following bacteria: Escherichia coli (gene: glpG, BVECGG), Providencia stuartii (gene: A55862), Pseudomonas aeruginosa (gene: B83259), Thermotoga maritima (genes: AAD36164 and AAD35669), Bacillus subtilis (genes: ydcA, G69772 and yqgP, BAA12519), Bacillus halodurans (gene: BAB05140), Pyrococcus horikoshii (gene: E71025), and Aquifex aeolicus (gene: AAC07308).

Using the standard COS cell cleavage assay (but transfecting 100ng of Rhomboid DNA per 35mm well) rhomboids from E coli, Providencia and Pseudomonas (all of which are human pathogens) and B. subtilis yqgP (gram positive) have been observed to cleave the Drosophila substrates Spitz, Gurken and Keren.

Thus functional Rhomboid polypeptides may be identified by bioinformatic techniques. Even bacterial rhomboids, which are more than a billion years diverged from humans and flies, have

the same core catalytic activity; they are intramembrane proteases. This demonstrates that Rhomboids are a functional enzyme family that share the same core activity of intramembrane serine proteases. Furthermore, substrate specificity has been conserved between Drosophila Rhomboids 1-4, human RHBDL2 and a number of bacterial rhomboids.

Yeast Rhomboid Polypeptides

Saccharomyces cerevisiae Rhomboid polypeptides YGR101w and YPL246c were cloned and their function investigated. YGR101w regulates mitochondrial function while YPL 246c appears to be involved in endocytosis.

Yeast Rhomboid-1 (YGR101w)

Expression of GFP-tagged Yeast Rhomboid-1 and co-staining with mitochondrial markers indicates that the protein is expressed not in the Golgi apparatus but in the mitochondria. This was also predicted from the Yeast Rhomboid-1 sequence using MITOPROT (Claros & Vincens,1996, Eur J. Biochem. 241,779-786).

Deletion of ygr101w causes slow growth and morphological disruption of mitochondria, as indicated by EM analysis and fluorescent staining with mitochondrial markers. Moreover, deletion mutants fail to grow in conditions in which glycerol is the only carbon source. This is a classic sign of disruption of respiratory metabolism.

Our results indicate that these phenotypes are caused by the absence of YGR101w serine protease activity because replacement of the wild type gene with a catalytically dead form (GAGG instead of GASG around the active serine) fails to rescue the cell, while replacement with a wild-type form does successfully rescue the cell.

The substrate for yeast Rhomboid-1 may be one or more of the following, which are all single TMD mitochondrial proteins with single TMDs, which act as soluble, cleaved proteins:

i) PET100/YDR079W-protein involved in assembly of cyt c oxidase,

ii) OSM1/YJR051W -oxidoreductase protein involved in osmolarity regulation,

iii) MGM1/YOR211C-dynamin related protein involved in membrane fusion,

iv) MCR1/YKL150W-oxidoreductase protein involved in oxidative stress resistance,

v) CCP1/YKR066C-oxidoreductase protein involved in cell stress.

Significantly, MGM1 and PET100 mutants share the YGR101W phenotype, making these favoured candidates for YGR101w substrates.

Yeast Rhomboid-2 (YPL246c)

Yeast Rhomboid-2 (YPL246c) is expressed in the secretory pathway and its deletion impairs membrane and vesicle dynamics. Electron microscopy indicates that YPL246c knockouts have extraneous membrane fragments in their cytoplasm. Furthermore, although in the wild-type the SNARE SNC1 recycles between the Golgi apparatus and the plasma membrane via endosomes (Lewis M. et al (2000) Mol. Cell. Biol. 11 23-28), this recycling is disrupted in the knockouts.

The uptake of the fluorescent dye FM4-64 (Vida and Emr (1995) J. Cell Biol. 128 779-792) is also impaired in knockouts.

This evidence indicates a defect in endocytosis, although other aspects of the secretory/endocytic pathway could be also disrupted. The mutant phenotype can be rescued by Knock-in of

the wild-type YPL246c gene but not by a catalytically dead form (GASG to GAGG). This demonstrates that its function is dependent on the Rhomboid-like intramembrane serine protease activity.

Zebrafish (*Danio rerio*) RHBDL2

To investigate the role of vertebrate Rhomboids, the zebrafish homologue of human RHBDL2 (sequenced from cDNA clone 2652120, GenBank accession number AW422344) was knocked out using the standard technology of antisense morpholino oligonucleotides (reviewed in Heasman J. Dev Biol. 2002 Mar 15;243(2):209-14), which allows the rapid removal of a gene function. Zebrafish embryos are a common model system as their embryonic development is easily observed.

RHBDL2 was observed to be expressed in a dynamic and specific expression pattern during embryogenesis. Knockout with the anti-sense morpholino oligo TCTTGCTCTTCGGTGTCATTATCGC leads to specific defects in the brain, the otic placode and the tail. These regions correspond to sites of RHBDL2 gene expression.

These results provide the first indication of a significant, non-redundant function of vertebrate rhomboids and indicate that other vertebrate rhomboids, in particular mammalian rhomboids, will also participate in physiologically-significant processes.

Active Form of Rhomboid-1

All known intramembrane proteases are synthesised as inactive zymogens which are activated by endoproteolytic cleavage.

Analysis of tagged Rhomboid-1 revealed that the predominant form in cells was full length, as estimated by apparent

molecular weight, but a cleaved form was also apparent in both COS and S2 cells.

The fact that the full length form was the predominant form was established by demonstrating that western blots with anti-HA (the epitope tag) of N and C-terminal tagged forms showed the same, full-length product – the only band that these tagged forms could have in common is the full length protein.

The size of the proteolytic fragment was compared to a set of truncated versions of Rhomboid-1, and this showed that the cleavage occurred within the lumenal loop between TMD 1 and 2.

Mutations in conserved amino acids in the cytoplasmic regions of TMDs 2 and 3 abolished this cleavage, but these non-cleavable proteins had full Spitz proteolytic activity, demonstrating that the full length forms of Rhomboid-1 are active.

Mutation of Rhomboid-1 active site residues abolished its protease activity, but did not affect the endoproteolysis of Rho itself, showing that this cleavage is not autocatalytic (i.e. Rhomboid-1 activity is not responsible for its own cleavage).

These results show that full-length Rhomboid-1 protein is active and, unlike other intramembrane proteases, it does not require proteolytic activation, either by its own activity or by other proteases. This has practical advantages in producing active enzyme in vitro, as simple expression of the protein is sufficient for activity.

Function of Star

In the absence of Star, Spitz is retained in the ER. This explains why the domain of EGF receptor activation is much narrower than the expression pattern of Spitz (Rutledge et

al., (1992) Genes Dev. *6*, 1503-1517; Gabay et al., (1997) Science *277*, 1103-1106), and why ectopic expression of full-length Spitz does not activate the receptor (Schweitzer et al., 1995 supra). Star, a protein with a single TMD (Kolodkin et al., (1994) Development *120*, 1731-1745), is necessary to translocate Spitz into the Golgi apparatus. The principal interaction between Spitz and Star occurs between the lumenal domains of the two proteins and this interaction counteracts the cytoplasmic Spitz ER retention.

Star does not act by specifically blocking the ER retention signal which is present in the Spitz cytoplasmic domain. Two chimeras containing the Spitz lumenal domain with the human TGFα C-terminal domain are not held in the ER in COS cells, but are nevertheless re-localised by Star. This provides indication that Star actively exports Spitz from the ER.

Drosophila genetics indicates that Star and Rhomboid-1 are both prime regulators of EGF receptor activity: they both appear to be necessary and they cannot replace each other (Guichard et al.,(1999) Development *126*, 2663-76). It has not been possible until now to separate their functions. Our results explain their co-dependency and synergy, and also provide a clear mechanistic distinction between Star and Rhomboid-1.

Star is not necessary for Rhomboid-1-dependent proteolysis itself, as an enzymatic cofactor. The Spi:TGFα-C chimera leaves the ER independently of Star and can be cleaved by Rhomboid-1 in the absence of Star. Therefore the sole function of Star in the activation of Spitz is to chaperone it from the ER to the Golgi apparatus, thereby delivering it to Rhomboid-1.

Optimised Substrate Design

To simplify and optimize assays using Rhomboid polypeptides, a chimeric substrate was designed to be cleaved more efficiently than any of the natural substrates and have a broad specificity for a range of rhomboids that cleave Drosophila Spitz (e.g. all Drosophila rhomboids, human RHBDL2, E. coli, Providencia and Pseudomonas Rhomboid).

The TGFα-GFP-Spi-TGFα 'ideal substrate' construct (here termed ST) was constructed using standard techniques from the following (nucleotide coordinates): 1-34 is TGFα UTR, 35-130 (35 is first A of ATG) is TGFα signal/propeptide sequence up to the BsiWI site (which we engineered into the sequence and into which any tag can be cloned) then GFP, and the remainder is TGFα with the Spitz 15aa and TMD (1045-1159). This insert was cloned into the pcDNA3.1(+) vector with HindIII (5') and Xbal (3').

The ST substrate is cleaved very efficiently in mammalian cells and is secreted very efficiently. This is significant because several known substrates can be cleaved but are not then efficiently released into the medium and secretion is an important requirement for any high throughput assay. The ST substrate is Star-independent (since its cytoplasmic domain derives from human TGFα), which provides a simpler and more direct assay and is tagged to provide for assay automation.

A convenient restriction site (BsiWI) has been engineered in the N-terminal domain of the constructs used in the present experiments into which tags, such as GFP, luciferase and alkaline phosphatase, can readily be introduced by standard techniques. The presence of tags at this position does not impair the cleavage of the substrate.

A vector comprising the ST coding sequence is co-transfected into mammalian cells (e.g. COS or HeLa) with an appropriate amount of the DNA encoding the Rhomboid to be screened. The accumulation of a soluble form of the tagged N-terminal domain can then be measured in the supernatant of the cells.

Since this cleaved fragment is tagged (e.g. with GFP, luciferase or alkaline phosphatase) the detection is readily automated. Other detection methods may also be used (e.g. ELISA, western blot, radioimmunoassay etc).

This assay is performed in the presence and absence of test compounds and the cleavage efficiency is compared.

The assay may be performed using stable lines of cells which express some or all of the components (i.e. rhomboid and substrate).

As we have shown herein, rhomboid has a very high activity; reducing its concentration by ten-fold or more may actually increase its cleavage ability (as it becomes less toxic to the cell), and as low as 0.025ng of DNA per transfection (in a 35mm well) has been observed to give detectable activity.

An assay method employing typical amounts of input DNA (e.g. 250ng per 35mm well), may put the amount of Rhomboid in cells into large excess and even inhibition of >90% might be missed. Suitable concentrations of Rhomboid are rate-limiting so that the effect of inhibitors (or activators) can be detected.

This can be readily determined individually for any particular assay using routine methodology but the present data indicates that a suitable starting point for optimization would be

around 0.25ng of Rhomboid DNA transfected into the number of cells used for a 35mm well.

Factors that might alter this level include the activity of the specific rhomboid being tested, its expression levels in the cells being used and driven by the promoter being used; the sensitivity of the detection system; and the transfection efficiency.

Function of Rhomboid

Rhomboid-1 is a Golgi-localised protein that is responsible for the proteolytic cleavage of Spitz. Moreover, since in the presence of Star and Rhomboid-1, Spitz accumulates in the Golgi apparatus, Rhomboid-dependent cleavage is the rate limiting step in the production of active Spitz and thereby EGF receptor activation.

Star and Rhomboid-1 are sufficient to cause efficient Spitz cleavage in all mammalian cell lines tested, providing indication that they are the only components required for Spitz cleavage. Since our analysis rules out the involvement of metalloproteases in Spitz processing, this further indicates that Rhomboid-1 may itself be the protease. The absence of a genetically identified candidate protease, other than Rhomboid-1, despite much genetic screening, is also consistent with this finding.

Confirmation of the protease activity of Rhomboid-1 is provided by mutagenesis analysis of conserved residues. This demonstrates that the Rhomboids are a family of novel intramembrane serine proteases, which is strongly supported by the observation that the Rhomboid-1 dependent cleavage of Spitz is sensitive to serine protease inhibitors. The four Drosophila Rhomboids tested all show distinct cleavage activities against three Drosophila EGFR ligands: Spitz,

Gurken and Keren (see Table 2), indicating the specificity of their serine protease.

The Spitz TMD (i.e. residues 141-144) is sufficient to confer Rhomboid-1 sensitivity onto TGFα and the actual site of cleavage and/or recognition is within this Spitz membrane spanning domain.

Because Rhomboid is highly conserved in many species, the elucidation of the EGFR ligand signalling mechanism, as described herein finds significant application in many fields of biology and medicine.

Accession	Gene	Size	Species
P20350	Rhomboid-1		Drosophila Melanogaster
AAK06753	Rhomboid-3		Drosophila Melanogaster
AAK06752	Rhomboid-2		Drosophila Melanogaster
CAA76629(XM_007948, NM_003961,AJ272344)	Rhomboid related protein (RHBL)	438	Homo Sapiens
AAK06754	Rhomboid-4		Drosophila Melanogaster
NP_060291	FLJ20435	292	Homo Sapiens
T16172	F26F4.3	419	C. elegans
AAA02747	AAA02747	325	Saccharum hybrid cultivar H65-7052
S40723	Rhomboid homlog C489B4.2	397	C. elegans
AAF88090	C025417_18	302	Arabidopsis thaliana
AAG51610	C010795_14	317	Arabidopsis thaliana
AAD55606	C008016_16	309	Arabidopsis thaliana
CAB88340	CAB8830	361	Arabidopsis thaliana
AAG28519	PARL	379	Homo sapiens
AE003628	CG5364/Rhomboid-5	1840	Drosophila melanogaster
CAB87281	CAB87281	346	Arabidopsis thaliana
T36724	T36724	297	Streptomyces coelicolor
A55862	AarA	281	Providencia stuartii
BAA12519	YpgP	507	B. subtilis
AAF53172	CG17212/Rhomboid-6	263	Drosophila melanogaster
BAB05140	BH1421	514	Bacillus halodurans
T02735	T9I4.13	372	Arabidopsis thaliana
CAA17304	Rv0110	249	Mycobacterium tuberculosis
T34718	T34718	383	Streptomyces coelicolor
BAB21138	BAB21138	393	Oryza sativa
AAD36164	E001768_13	222	Thermatoga maritime
AAD35669	AE001733_6	235	Thermatoga maritime
T35521	T33521	256	Streptomyces coelicolor
CAC18292	CAC18292	497	Neurospora crassa
T05139	F7H19.260	313	Arabidopsis thaliana
AAG40087	AC079374_1	369	Arabidopsis thaliana
B75109	PAB1920	212	Pyrococcus abyssi
AAK04268	AE006254_9	230	Lactococcus lactis
CAA76716	CAA76716	164	Rattus norvegicus
AAF58598	CG8972/Rhomboid-7	351	Drosophila melanogaster
CAA86933	CAA86933	276	Acinetobacter calcoaceticus
CAA97104	YGR101w/Yeast Rhomboid-1	346	Saccharomyces cerevisiae

AAC07308	AAC07308	227	Aquifex aeolicus
E72574	APE1877	256	Aeropyrum pernix
NP_069844	NP_069844	330	Archaeoglobus fulgibus
AAA58222	AAA58222	274	E. coli
BVECGG	GlpG	276	E. coli
E71025	PH1497	197	Pyrococcus horikoshii
AAK03522	GlpG	291	Pasteurella multocida
G82780	XF0649	224	Xylella fastidiosa
G69772	YdcA	199	Bacillus subtilis
O14362	C30D10.19C	298	Schizosaccharomyces pombe
F82729	XF1054	232	Xylella fastidiosa
BAB04236	BH0517	248	Bacillus halodurans
T34866	T34866	285	Streptomyces coelicolor
A82363	GlpG	277	Vibrio cholerae
I64081	GlpG	192	Haemophilus influenzae
AC026238	AC026238	336	Arabidopsis thaliana
AAH03653	AAH03653	329	Homo sapiens
D71258	GlpG	208	Treponema pallidum
CAB9075	CAB9075	223	Streptococcus uberis
AAK24595	AAK24595	218	Caulobacter crescentus
B83259	PA3086	286	Pseudomonas aeruginosa
C82588	XF2186	206	Xylella fastidosa
AAG19304	Vng0858c	598	Halobacterium sp.NRC-1
BAB02051	MKP6.17	506	Arabidopsis thaliana
AAG18926	Vng0361c	333	Halobacterium sp.NRC-1
BAB29735	BAB29735	315	Mus musculus
E75328	E75328	232	Deinococcus radiodurans
T49293	T16L24.70	269	Arabidopsis thaliana
CAB83168	CAB83168	392	Schizosaccharomyces pombe
T45666	F14P22.50	411	Arabidopsis thaliana
P53426	B1549_C3_240	251	Mycobacterium leprae
CAC22904I	CAC22904I	214	Sulfolobus solfataricus
T41608	SPCC790.03	248	Schizosaccharomyces pombe
H81375	Cj1003c	172	Campylobacter jejuni
CAC31552	CAC31552	238	Mycobacterium leprae
Q10647	YD37_MYCTU	240	Mycobacterium tuberculosis
NP_015078	Yp1246cp	262	Saccharomyces cerevisae
S76748	S76748	198	Synechocystis sp.
NM_017821	RHBDL2		Homo sapiens
BE778475	RHBDL3 (partial)		Homo sapiens

Table 1 Rhomboid polypeptides

Accession	Name	Size	Species
Q01083	Spitz	230	D. melanogaster
AAF63381	Keren/Gritz/Spitz-2	217	D. melanogaster
P42287	Gurken	294	D. melanogaster
P01135	TGF-α	160	Homo sapiens
P00533	EGF	1210	Homo sapiens
Q99075	HB-EGF	208	Homo sapiens
JC1467	Betacellulin	178	Homo sapiens
A34702	Amphiregulin	252	Homo sapiens
BAA22146	Epiregulin	169	Homo sapiens
Q03345	Lin-3	438	C. elegans

Table 2

Name	WT Rho-1 sequences present in construct
N1	1-101
N2	1-122
N3	1-135
N4	1-164
N5	1-210
N6	1-236
N7	1-299
N8	1-328
C5	208-355
C4	162-355
C3	151-355
C2	136-355
C1	120-355

Table 3

CLAIMS:

1. An assay method for identifying a modulator of a Rhomboid polypeptide, which method comprises:
(a) bringing into contact an Rhomboid polypeptide and a polypeptide substrate in the presence of a test compound; and
(b) determining proteolytic cleavage of the polypeptide substrate.

2. An assay method according to claim 1 wherein the Rhomboid polypeptide has a sequence shown in Table 1.

3. An assay method according to claim 2 wherein the Rhomboid polypeptide is selected from the group consisting of Drosophila Rhomboid 1, Drosophila Rhomboid 2, Drosophila Rhomboid 3, Drosophila Rhomboid 4, Human RHBDL-1, Human RHBDL-2 and Human RHBDL-3, E. coli glgG, B. subtilis ypqP, P. stuartii A55862 gene product, P. aeruginosa B83259 gene product, S. cervisiae YGR101w and S. cervisiae YPL246c.

4. An assay method according any one of the preceding claims wherein the Rhomboid polypeptide comprises an ER (endoplasmic reticulum) retention signal.

5. An assay method according any one of the preceding claims wherein the polypeptide substrate is an EGFR ligand.

6. An assay method according to claim 5 wherein the polypeptide substrate has a sequence shown in Table 2.

7. An assay method according to any one of the preceding claims wherein polypeptide substrate comprises a detectable label.

8. An assay method according to any one of the preceding claims comprising identifying said test compound as a modulator of Rhomboid protease activity.

9. An assay method according to claim 8 further comprising determining the ability of said test compound to inhibit the infectivity of a microbial pathogen.

10. An assay method according to claim 8 or claim 9 comprising isolating said test compound.

11. An assay method according to claim 10 comprising formulating said test compound in a pharmaceutical composition with a pharmaceutically acceptable excipient, vehicle or carrier.

12. A modulator of Rhomboid protease activity obtained by a method of any one of claims 1 to 10.

13. A modulator according to claim 12 comprising a peptide fragment of a Rhomboid polypeptide.

14. An EGFR ligand polypeptide which is proteolytically cleaved by a Rhomboid polypeptide and which comprises the transmembrane domain of a first EGFR ligand and the intracellular and extracellular domains of a second EGFR ligand.

15. An EGFR ligand polypeptide according to claim 14 wherein the first polypeptide is Spitz and the second polypeptide is TGFα.

16. An EGFR ligand polypeptide according to claim 14 or claim 15 wherein the extracellular domain further comprises a detectable label.

17. An isolated nucleic acid encoding a Rhomboid polypeptide which comprises the amino acid sequence shown in Figure 8, Figure 10 or a fragment thereof.

18. An isolated nucleic acid according to claim 17 comprising the nucleic acid sequence of Figure 7, Figure 11 or a fragment thereof.

19. An isolated nucleic acid having greater than about 55% sequence identity with the nucleic acid sequence of Human RHBDL3 as shown in figure 7 or Zebrafish RHBDL shown in figure 10.

20. An isolated nucleic acid that hybridizes with the nucleic acid sequence shown in figure 7 or figure 10 under stringent conditions.

21. An isolated Rhomboid polypeptide encoded by a nucleic acid sequence according to any one of claims 17 to 20.

22. An isolated Rhomboid polypeptide having greater than about 70% sequence identity with the amino acid sequence of Human RHBDL3 shown in Figure 8 or Zebrafish RHBDL2 shown in figure 11.

23. A Rhomboid polypeptide fragment comprising 325 amino acids or less which proteolytically cleaves a polypeptide substrate.

24. A Rhomboid polypeptide fragment according to claim 23 wherein the polypeptide substrate is an EGFR ligand shown in Table 2.

25. A Rhomboid polypeptide fragment according to claim 23 or claim 24 comprising amino acid residues R152, G215, S217 and H281 of the Drosophila Rhomboid-1 sequence.

26. A Rhomboid polypeptide fragment according to claim 25 wherein the Rhomboid polypeptide is selected from the group consisting of Drosophila Rhomboid 1, Drosophila Rhomboid 2, Drosophila Rhomboid 3, Drosophila Rhomboid 4, Human RHBDL-1, Human RHBDL-2 and Human RHBDL-3, E. coli glgG, B. subtilis ypqP, P. stuartii A55862 gene product, P. aeruginosa B83259 gene product, S. cervisiae YGR101w and S. cervisiae YPL246c.

27. A Rhomboid polypeptide which proteolytically cleaves a polypeptide substrate and which comprises a heterogeneous endoplasmic reticulum retention signal.

28. A Rhomboid polypeptide according to claim 27 wherein the endoplasmic reticulum retention signal is KDEL.

29. A Rhomboid polypeptide according to claim 27 or claim 28 wherein the polypeptide substrate is an EGFR ligand shown in Table 2.

30. A Rhomboid polypeptide according to any one of claims 27 to 29 comprising amino acid residues R152, G215, S217 and H281 of the Drosophila Rhomboid-1 sequence.

31. A Rhomboid polypeptide according to claim 30 wherein the Rhomboid polypeptide is selected from the group consisting of Drosophila Rhomboid 1, Drosophila Rhomboid 2, Drosophila

Rhomboid 3, Drosophila Rhomboid 4, Human RHBDL-1, Human RHBDL-2 and Human RHBDL-3, E. coli glgG, B. subtilis ypqP, P. stuartii A55862 gene product, P. aeruginosa B83259 gene product, S. cervisiae YGR101w and S. cervisiae YPL246c.

32. An isolated nucleic acid encoding a Rhomboid polypeptide fragment according to any one of claims 23 to 26 or a Rhomboid polypeptide according to any one of claims 27 to 31.

33. A recombinant vector comprising a nucleic acid according to any one of claims 17 to 20 or claim 32.

34. A host cell comprising a recombinant vector according to claim 33.

35. A method of producing a Rhomboid polypeptide comprising:
(a) causing expression from nucleic acid which encodes a Rhomboid polypeptide in a suitable expression system to produce the polypeptide recombinantly;
(b) testing the recombinantly produced polypeptide for Rhomboid protease activity.

36. A method of obtaining a substrate for a Rhomboid polypeptide comprising,
(a) providing a test polypeptide,
(b) bringing into contact an Rhomboid polypeptide and the test polypeptide under conditions in which the Rhomboid polypeptide normally catalyses proteolytic cleavage of a substrate; and,
(c) determining cleavage of the test polypeptide.

37. A method according to claim 36 wherein the test polypeptide comprises the amino acid sequence IASGA.

38. A method according to claim 37 wherein the test polypeptide comprises the amino acid sequence ASIASGA.

39. A method for proteolytically cleaving the transmembrane domain of a polypeptide comprising;
contacting the polypeptide with a Rhomboid polypeptide.

40. Use of a Rhomboid polypeptide for the proteolytic cleavage of the transmembrane domain of a polypeptide substrate.

41. A pharmaceutical composition comprising a polypeptide according to claim 21 or claim 22, a polypeptide fragment according to any one of claims 23 to 26 or a modulator according to claim 12 and a pharmaceutically acceptable excipient, vehicle or carrier.

42. Use of a polypeptide according to claim 21 or claim 22, a polypeptide fragment according to any one of claims 23 to 26 or a modulator according to claim 12 in the manufacture of a composition for treatment of a condition associated with aberrant ErbB or EGF receptor activity or a pathogen infection.

43. A method comprising administration of a composition according to claim 41 to a patient for treatment of a condition associated with aberrant ErbB or EGF receptor activity or a pathogen infection.

44. A method according to claim 43 wherein the condition associated with aberrant ErbB or EGF receptor activity is cancer, coronary atherosclerosis, psoriasis, wound healing, infant prematurity or a peripheral nerve injury/neuropathy.

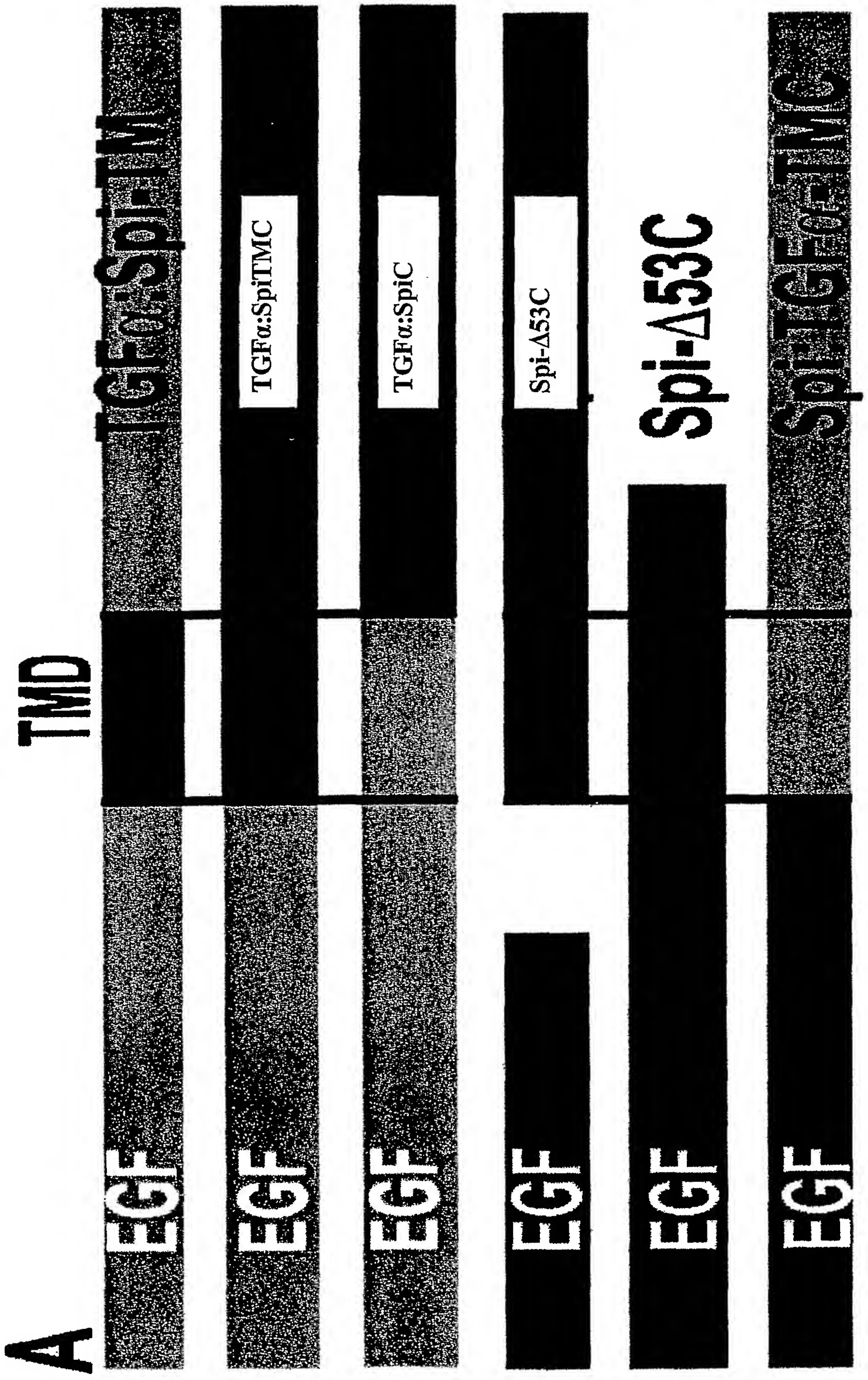

A
EGF
EGF
EGF
EGF
EGF
EGF
TMD
TGFα:SpiTMC
TGFα:SpiC
Spi-Δ53C
Spi-Δ53C
TGFα:SpiTMC
TGFα:SpiC
Spi-Δ53C

Figure 1

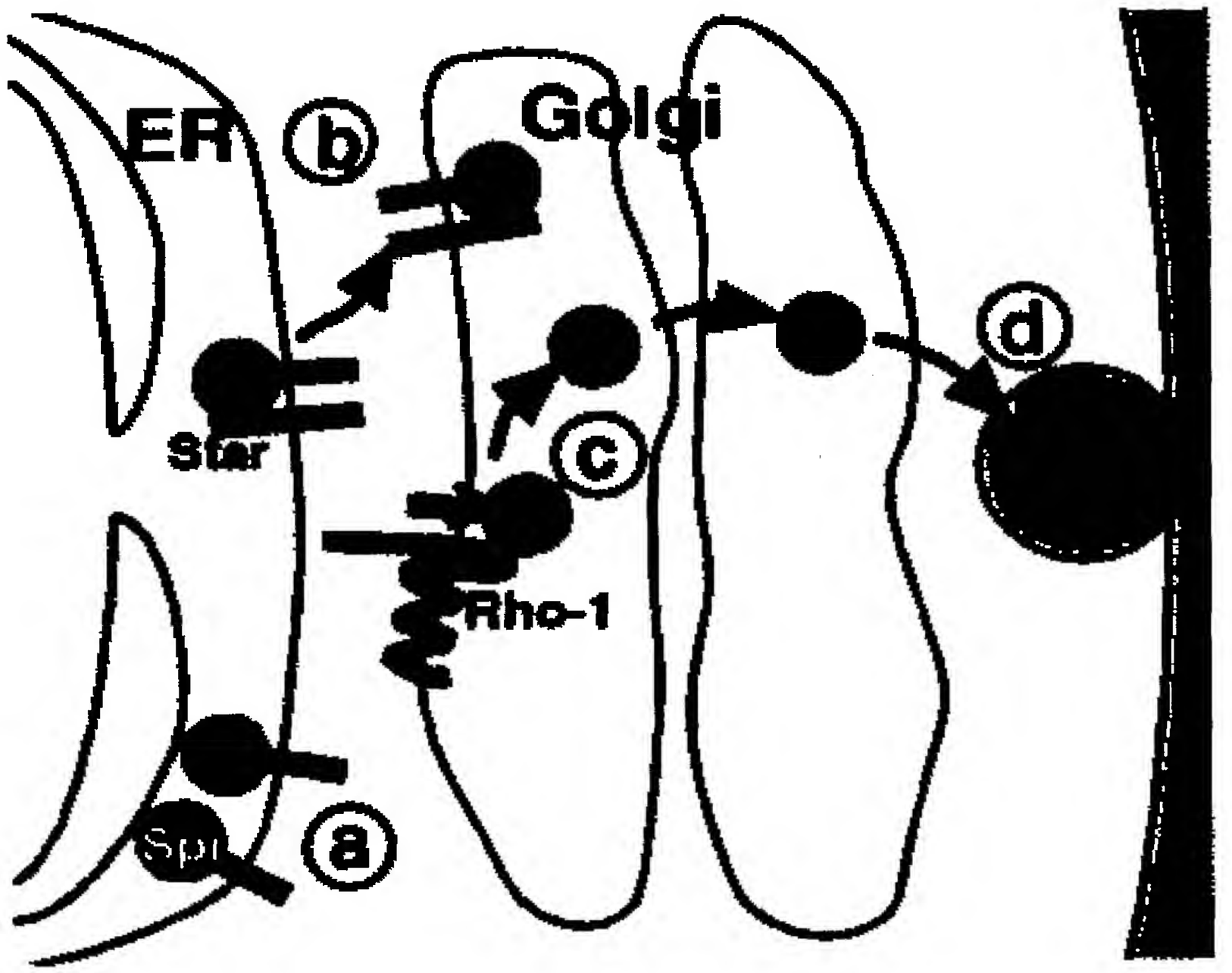


Figure 2

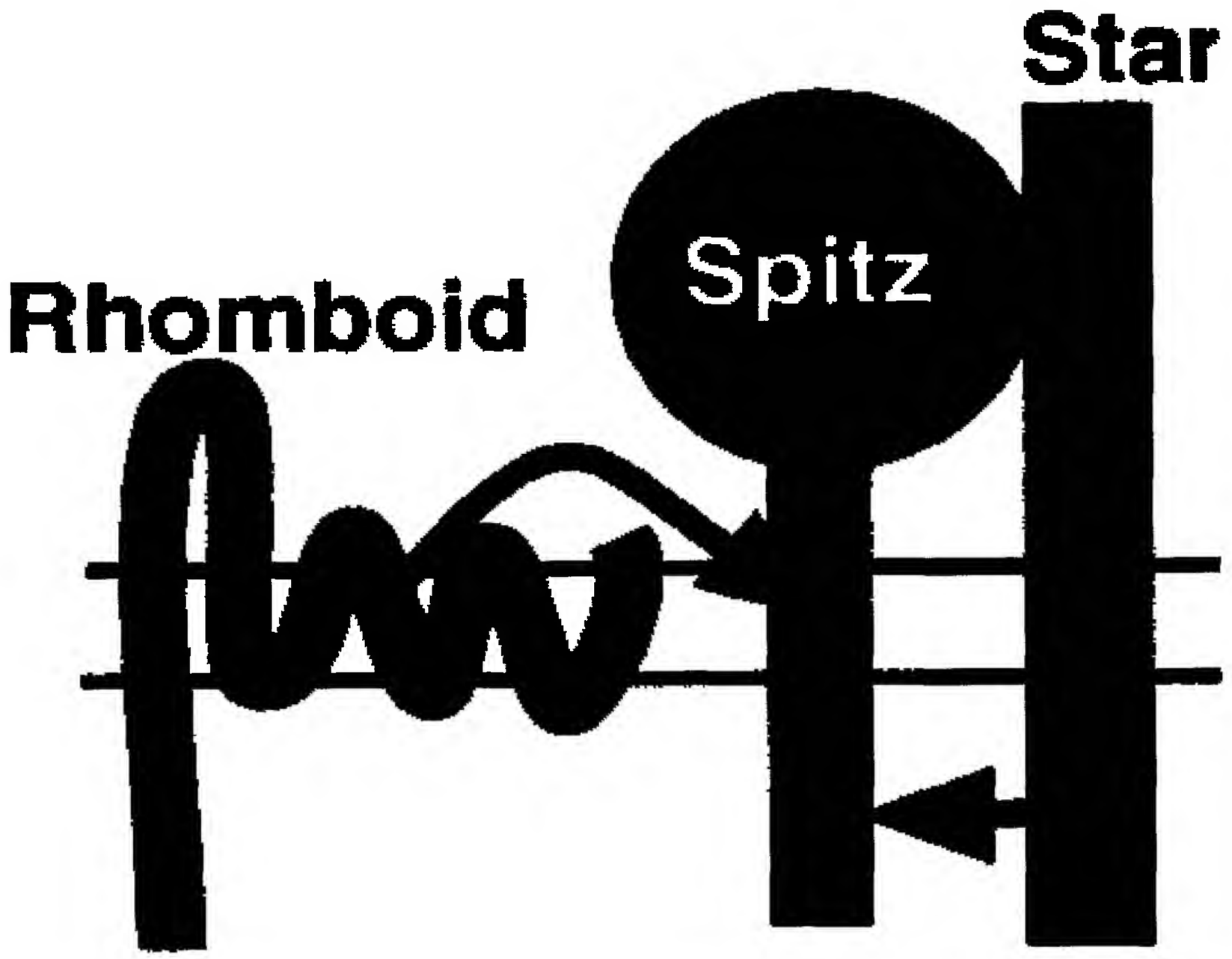


Figure 3

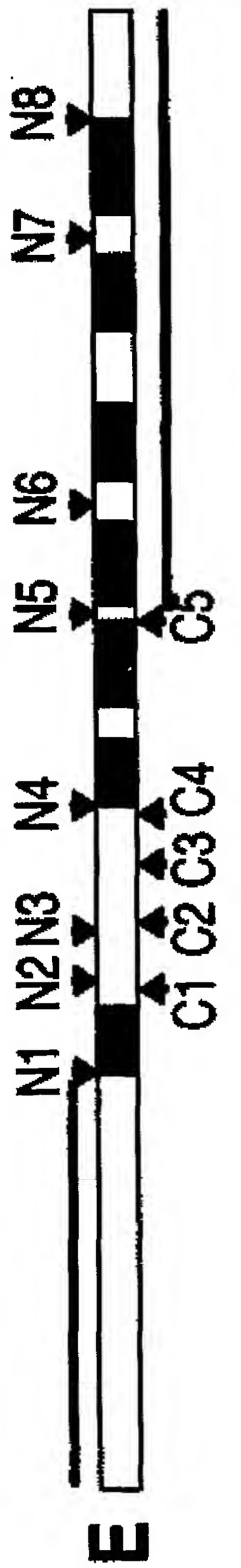
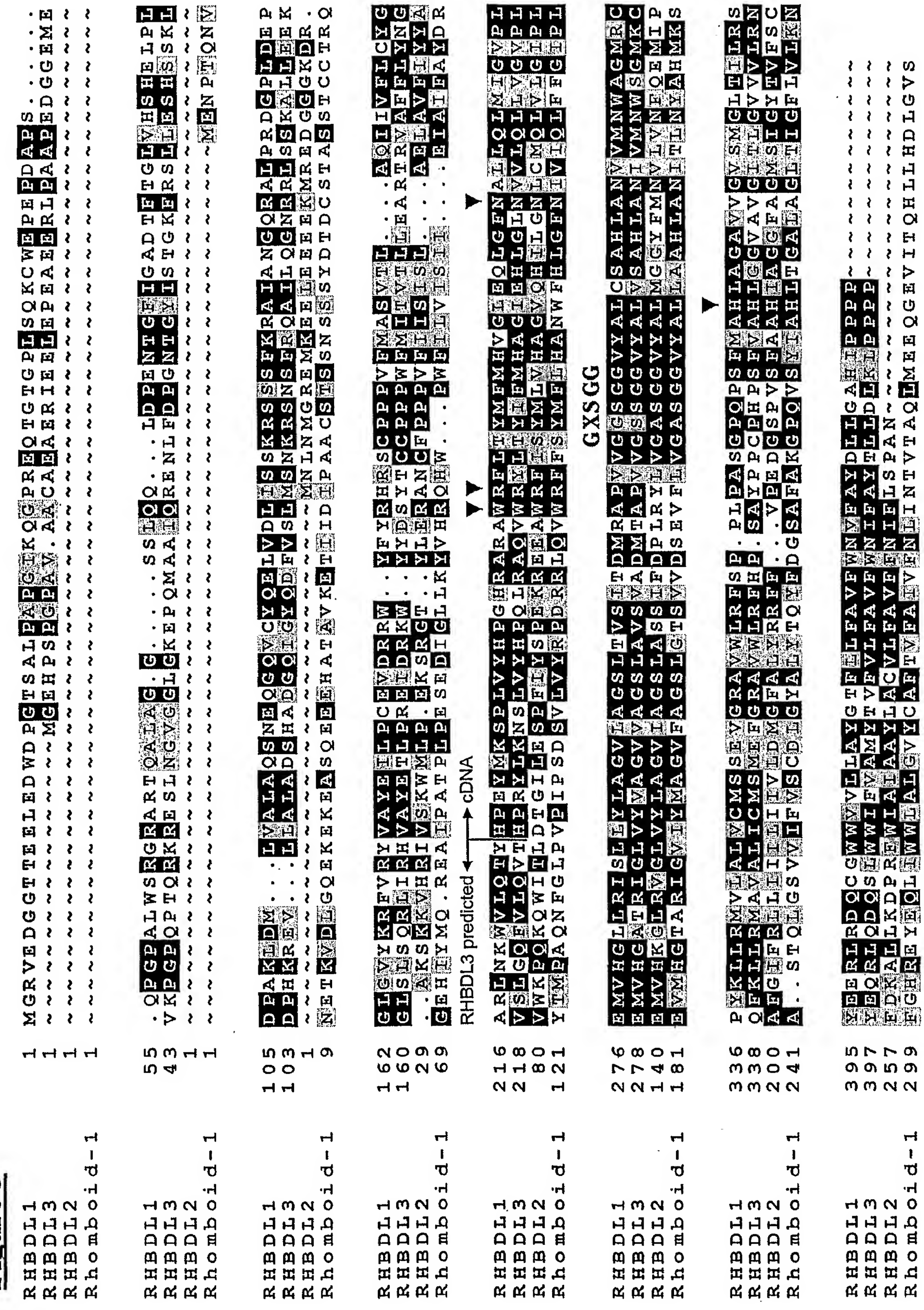


Figure 4

Figure 5



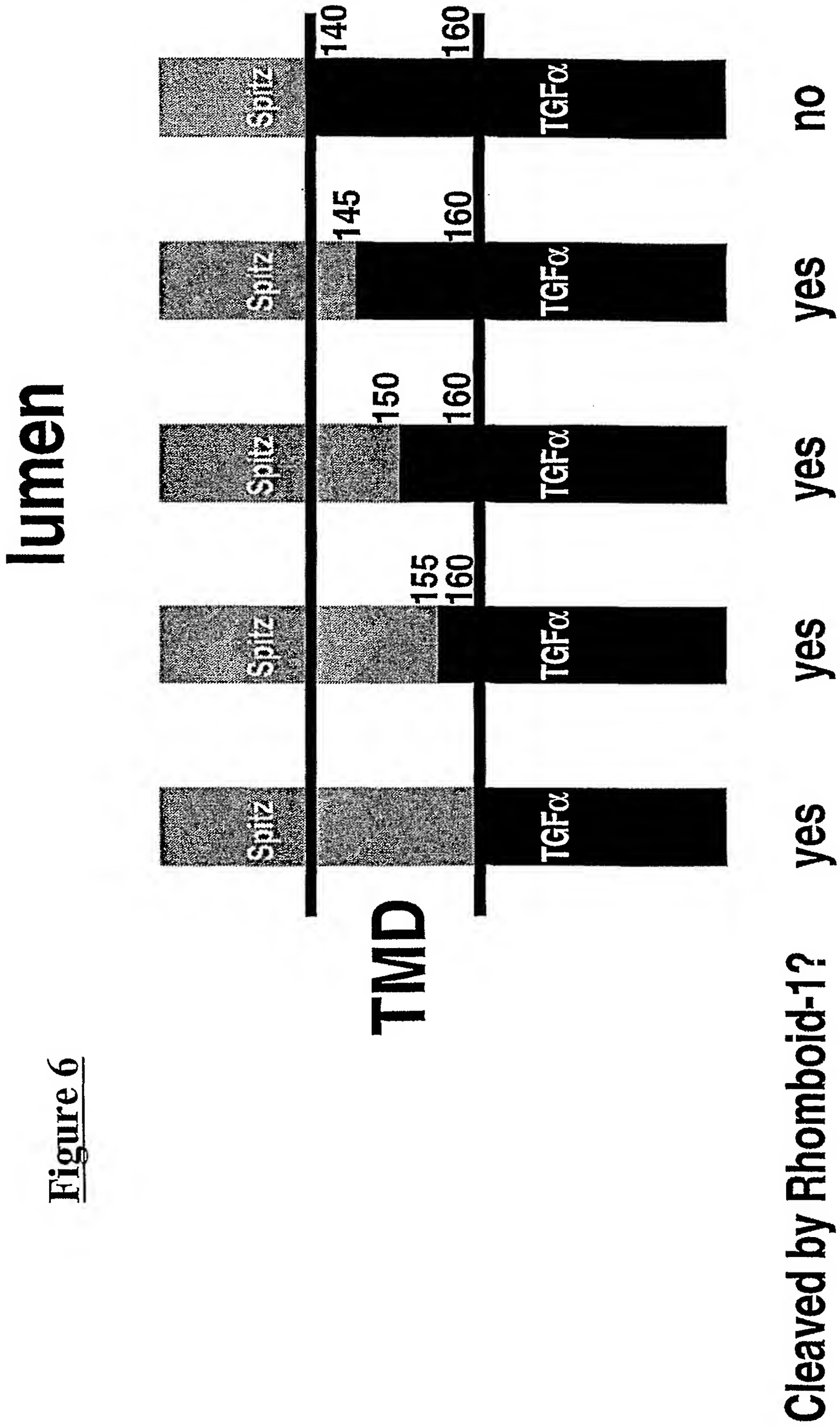

lumen
TMD
cytoplasm
Spitz
TGFα
140
160
145
160
150
160
155
160
yes
yes
yes
yes
no
Cleaved by Rhomboid-1?

Figure 6

atgggcgagcaccccagcccgggccccgcggtggccgcctgcgccgaggcggagcgcat

cgaggagctggaacccgaggccgaggagcggctgcccgcggcgccggaggacGGTGGGG

AGATGGAAGTGAAACCAGGCCCCCAACCCACACAACGAAAGCGGGAAAGTCTGAATGGG

GTTGGGGGGGctggggaaggagccccagatggcagcaatacaaagagagaatctgTTTGA

CCCTGGGAACACAGGCTACATTAGCACAGGCAAGTTCCGGAGTCTTCTGGAGAGCCACA

GCTCCAAGCTGGACCCGCACAAAAGGGAGGTCCTCCTGGCTCTTGCCGACAGCCACGCG

GATGGGCAGATCGGCTACCAGGATTTTGTCAGCCTAatgagcaacaagcgttccaacag

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TTTGGGCGGGCCGTGTGGCTCCGCTTCCACCCGTCGGCCTATCCCCCGTGCCCTCACCC

AAGCTTTGTGGCGCACTTGGGTGGCGTGGCCGTGGGCATCACCCTGGGCGTGGTGGTCC

TGAGGAACTACGAGCAGAGGCTCCAGGACCAGTCACTGTGGTGGATTTTTGTGGCCATG

TACACCGTCTTCGTGCTGTTCGCTGTCTTCTGGAACATCTTTGCCTACACCCTGCTGGA

CTTAAAGCTGCCGCCTCCCCCC ESTBE778475

Figure 7

MGEHPSPGPAVAACAEAERIEELEPEAEERLPAAPEDGGEMEVKPGPQPTQRKRESL
NGVGGLGKEPQMAAIQRENLFDPGNTGYISTGKFRSLLESHSSKLDPHKREVLLALA
DSHADGQIGYQDFVSLMSNKRSNSFRQAILQGNRRLSSKALLEEKGLSLSQRLIRHV
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HPRYLKNSLVYHPQLRAQVWRYLTYIFMHAGIEHLGLNVVLQLLVGVPLEMVHGA
TRIGLVYYVAGVVAGSLAVSVADMTAPVVGSSGGVYALVSAHLANIVMNWSGMKC
QFKLLRMAVALICMSMEFGRAVWLRFHPSAYPPCPHPSFVAHLGGVAVGITLGVV
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Figure 8

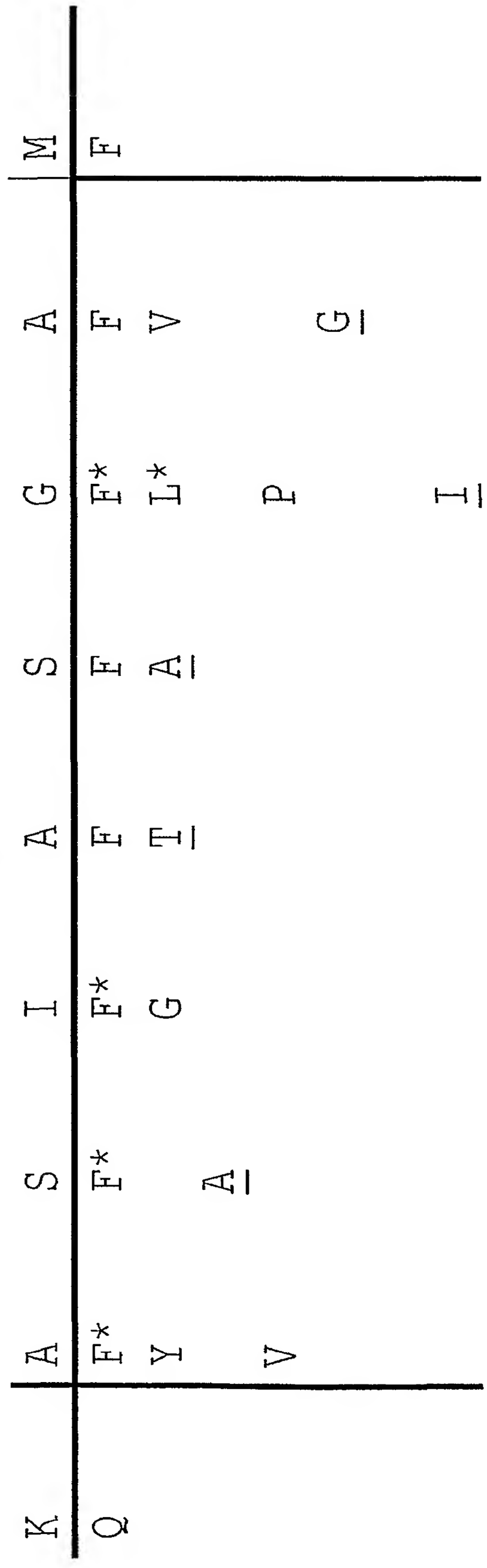

Figure 9

CACTGTTGGCCTACTGGGATGCCCCGCTAACAAATTCATGAATGGGAGTGAAGCAACGCTAC

TGACGCAGATAGAGAAATGGGCGATAATGACACCGAAGAGCAAGACTCTTTGCAGAAGAAGG

ACGAAGAAGCTGGTAACCGAGACAATCCGGTCAGAAGAGTTCGGAGGGTCGAGAAGTTTCAT

AAGAATGTTTCTAAATGGATGCTTCCCGAGGAGTTACATGAGACTTATCTTGAGCGGGCGAA

CTGCTGTCCGCCACCGATCTTCATCATCCTCATCAGTTTAGCAGAGCTGGCCGTGTTTATCT

ACTACGCTGTATGGAAGCCTCAAAAACAGTGGATAACTCTAGGAACTGGGATCTGGGATAGT

CCTCTTACCTATAGGCCAGAACAACGCAAGGAGGCTTGGCGCTTTGTTTCCTACATGTTTGT

ACATGCCGGGGTGGAGCATATCATGGGGAACCTATTAATGCAGCTTCTTCTGGGTATTCCTC

TGGAACTGGTCCATAAAGGCTTTGAAGTTGGCATGGTGTACATGTGTGGGGTCCTCGCAGGG

TCTCTGGCCAGCTCCATCTTTGATCCTTTCAGTGCTCTTGTGGGAGCTTCAGGTGGTGTTTA

TGCCCTTATGGGTGGCTACTTCATGAATGCCATTGTGAATTTCCGGGAGATGAGAGTTCTTC

TAGGAGTGTTTCGCATCTTAGTGATTGTTTTGATTGTTGGAACAGATGTTGGATTTGCTCTT

TATAGAAGGTTCATTGTCCACGAGGCTGGCCTAAAGGTCTCTTTTGTGGCTCATATTGGCGG

TGGCATAGCAGGCATGACCATTGGTTATGTGTTTTTCACCAACTACAATAAAGAGCTTCTAA

AAGACCCACGCTTCTGGATGTGCATTGTGGGATACATCGTCTTCTTACTGTTTGCAGTCATT

TTCAACATCTTCTTGTCCCCAGCACCCGCATGAGGTCATCAATGGACAGTCGAACCTTTTTT

TTATTTTATAAAAGAATGAGGTCAACACAACTGTCAGACAATCCTGTTGGTATTTATAGACT

CATAAAGGGTTAGTTCAACTGAAAACTCTGTATTGACCCATATTGTTCTTTCAGAAGTTCAT

CTTTGAAACACAAATGAAGATATTTTTAAATCAAGCCGAGCGATTTCGTTTCTTCTATTCAG

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TTTATGAATGAATAGATTTAATTTTGGTTTACATTTCAGAAATTTGGATTTGGAAATCTTTA

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TAAATTTTAGAAAACCCTTGAGGGTGAGGAATTTGTGAGTAAATTTTGATTTGAGGGTTAAC

TATCCCTTTAAAAAAAGAGGTTTCGTTTTGATACCAATAGAGGGCAGCATTGATCAGCATGT

GGGCATTGGAAGACACTGACCTATAAAAAGTAGGAAATTGTTAAATCAGTGCTAATGACATG

CATCTGTATTTACCCTACGTATTTGTCCCTAATTATCAAATCATTTATTTTCAGAAATGGGT

TTGGGTTTGGAATGTTTTGGCCATATAGAGGCCATATAGCCTTTTTTTATTTTTTTTTATGAAA

TAATAAAAAGAATTGTGCCAATGTTTT

Figure 10

MGDNDTEEQDSLQKKDEEAGNRDNPVRRVRRVEKFHKNVSKWMLPEELHETYLERANCCPPP

IFIILISLAELAVFIYYAVWKPQKQWITLGTGIWDSPLTYRPEQRKEAWRFVSYMFVHAGVE

HIMGNLLMQLLLGIPLELVHKGFEVGMVYMCGVLAGSLASSIFDPFSALVGASGGVYALMGG

YFMNAIVNFREMRVLLGVFRILVIVLIVGTDVGFALYRRFIVHEAGLKVSFVAHIGGGIAGM

TIGYVFFTNYNKELLKDPRFWMCIVGYIVFLLFAVIFNIFLSPAPA

Figure 11